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Reliability of 50% Egg Infectivity Titrations with Suspensions of Influenza Viruses Prepared from Mouse Lungs.* (20002)

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The reliability of replicate 50% end point determinations, employing the PR8 strain of influenza virus for titration of infectivity in mice or eggs, has previously been described (1,2). Weighted end points, based on pulmonary lesions, and EID_{50} determinations were shown to have approximately the same reproducibility, if equal numbers of mice or eggs were employed in each type of test. It is noteworthy that these studies were carried out with a strain of virus highly adapted to eggs and mice and that purified suspensions of egg passage virus were used in these experiments. The present report describes the precision of duplicate titrations of infectivity in eggs executed with crude suspensions of mouse lungs infected with recently isolated strains of Type A-prime and Type B influenza viruses. These lines of virus had been passed relatively few times in eggs or mice, and might be considered to be less well adapted than the PR8 strain for growth in either host. Moreover, a variable period of time extending up to 3 months intervened between the first and the second determination, and during this interval the lung suspensions were stored in the frozen state. Nevertheless, it will be shown that under these circumstances the reliability of 50% end point determinations in eggs was approximately equal to that described for similar determinations, in eggs or in mice, with purified suspension of the PR8 strain of virus.

Materials and methods. Viruses. The Rhodes strain of Type A-prime and the Allen

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TABLE I. Standard Error of the Difference between Duplicate Titrations (EID_{50}) with Influenza Viruses.

Strain	Type	Passage	1st titration	2nd titration	Diff. (Δ)	Diff. from the mean* ($\Delta-\bar{\Delta}$)	Square of diff. from the mean $(\Delta-\bar{\Delta})^2$
Allen	B	$F_1E_{19}M_1$	4	4.6	+.6	+.6	.36
		"	4.5	4.7	+.2	+.2	.04
		"	4.3	4	—.3	—.3	.09
		"	4.5	4.5	0	0	0
		$_{19}M_2$	5.3	5	—.3	—.3	.09
		"	5.3	4.5	—.8	—.8	.64
		$_{19}M_5$	4.7	4.2	—.5	—.5	.25
		$_{23}M_7$	3.3	3.5	+.2	+.2	.04
		$_{23}M_9$	5	4.5	—.5	—.5	.25
		$_{19}M_{11}$	4.7	4.7	0	0	0
		"	4.7	4.6	—.1	—.1	.01
		$_{19}M_{13}$	6.5	6.5	0	0	0
		"	6.7	6.7	0	0	0
		"	6.5	6.5	0	0	0
		$_{23}M_{13}$	3.5	4	+.5	+.5	.25
		$_{19}M_{19}$	5	5.6	+.6	+.6	.36
		"	6	6.5	+.5	+.5	.25
		$_{19}M_{21}$	6.2	6	—.2	—.2	.04
		F_1M_{22}	4	4.5	+.5	+.5	.25
Warner	B	$E_{11}M_1$	5.3	4.6	—.7	—.7	.49
		"	5	5.3	+.3	+.3	.09
		$E_{11}M_{10}$	7.5	6.7	—.8	—.8	.64
Rhodes	A′	F_4M_{22}	7	7	0	0	0
			7	7	0	0	0
			7	7.2	+.2	+.2	.04
			5.5	5.7	+.2	+.2	.04
			7	7	0	0	0
	Total = 27				—.4		4.22

* Mean difference between duplicate titrations $= \frac{-.4}{27} = -.015$, *i.e.* $0 = \bar{\Delta}$.

Stand. error of the difference $= \sqrt{\Sigma\frac{(\Delta-\bar{\Delta})}{N-1}} = \sqrt{\frac{4.22}{26}} = .40.$

strain of Type B influenza virus were isolated in this laboratory in 1947 and 1945, respectively. The Warner strain of Type B influenza virus was isolated in Australia in 1945 and obtained through the courtesy of F. M. Burnet. The passage history of each line is given by a formula in which the letters F, M, and E refer to ferret, mouse, or egg passage. The subscript refers to the number of transfers made, and the order of the letters refers to the sequence in which the passages were carried out. *Viral suspensions.* Lungs were harvested from mice aseptically at variable intervals after inoculation. Ten per cent suspensions were prepared by grinding lungs in a mortar with sterile alundum, employing 2 ml of diluent per lung. The suspensions were lightly centrifuged, the supernates were decanted and used, and the sediments were discarded. *Titrations of infectivity in eggs.* Serial 10-fold dilutions of virus suspensions were made in diluent chilled in an ice-water bath. A volume of 0.2 ml of the desired dilution of virus was inoculated in the allantoic cavity of fertile chicken eggs which had been incubated at 38°C for 9 or 10 days prior to inoculation. Four eggs were used for each dilution. In titrations with Type B influenza virus the eggs were incubated at 35°C for 72 hours before harvesting. With Type A-prime virus a 48-hour period of incubation was chosen. Allantoic fluid from each egg was tested for the presence of virus by agglutination of chicken erythrocytes, and the 50% infectivity titer (EID_{50}) was calculated(3). End points were calculated to two decimal places, and the values rounded to one decimal place. Titers are expressed as the logarithm of the reciprocal of the dilution calculated to represent one EID_{50}. *Solutions.* Saline refers to 0.15 M NaCl buffered at pH 7.2 with 0.01 M phosphate. Ten per cent normal horse serum in saline containing 600 units of penicillin and 1.25 mg of streptomycin per ml were used as diluent for the preparation of 10% suspensions of lung and for the dilution of lung suspensions used for titration. Normal horse serum was heated at 56°C for 30 minutes prior to use.

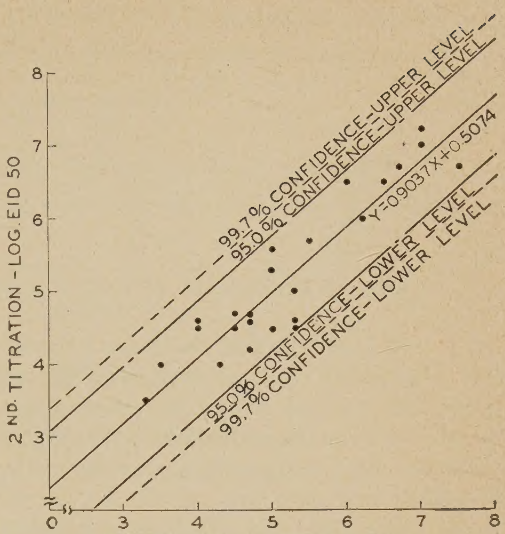

FIG. 1. Correlation of values observed in first determination and second determination.

Experimental. Standard error of the difference (S.E. diff.) between duplicate titrations. In the course of experiments designed for other purposes 27 duplicate titrations were performed with 24 different suspensions of mouse lung each containing one of 5 different lines of Type A-prime or Type B influenza viruses. These determinations were carried out by a single operator over a period of 3 years. In general, all suspensions had been stored in the frozen state at either —70°C or —50°C prior to titration. The results of these experiments and a tabulation of the calculations are shown in Table I. It is important to note that a difference in the end points obtained with virus suspensions are pertinent only when comparisons are made between rows in Table I (horizontal), as these figures represent the results of duplicate titrations. Differences within the columns (vertical) are attributed to the fact that either different viruses or different concentrations of virus were used as inocula, or that the lungs were harvested at different intervals of time after inoculation with the same suspension of virus. Inspection of the data indicates that the error of duplicate titrations was distributed normally. This impression was substantiated by demonstrating that if the differences between paired titrations were plotted on normal probability paper(4), a straight line relationship was evident. These findings validate the use of the Standard Error of the difference between duplicate titrations to assess the significance of variation between end points. The mean difference between duplicate titrations was —0.015. For practical purposes this value was considered as zero. The S.E. of the difference between duplicate titrations was 0.40. Thus variation between 2 end points of more than twice the S.E. Diff., *i.e.*, .80 may be considered statistically significant at the 95% level of confidence. Variation between 2 end points of more than 3 times the S.E. Diff., *i.e.*, 1.2 may be considered significant at the 99.7% level of confidence.

Analysis of the data by linear correlation. If the exact value of the first titration could be duplicated in the second, and if the values of the pairs of determinations were plotted on arithmetic grid so that ordinate distances represent values obtained in the first determination while abscissa distances those in the second, a straight line with a slope of one would be obtained on which all of the points would fall. An analysis of deviations from this theoretic ideal, in data derived experimentally, provides another measure of the reproducibility of these end points. In Fig. 1 the pairs of values recorded in Table I were plotted as described above. A line of best fit was calculated with equations derived from the theory of least squares(5). The slope was found to be 0.904. This value is not strikingly different from the theoretical slope of

TABLE II. Comparison of End Point Differences Required for Significance at Various Levels of Probability.

	End point difference required		
	Purified suspensions of Type A influenza virus		Crude suspensions of lungs infected with Type A′ and Type B influenza viruses
Probability (%)	Mouse titration* (5 mice)	Embryo titration† (5 eggs)	Embryo titrations (4 eggs)
95	.73	.62	.8
99.7	1.11	.93	1.1

* Recalculated from data of Lauffer & Miller(1).
† ” ” ” ” Knight(2).

one. Inspection of the deviations of the points from the line of best fit indicates that the error of these determinations is distributed normally. By a mathematical analysis of the deviations from this line of best fit, or expected value, one may calculate a standard error of the estimate (S.E. Est.)(6) of the agreement between duplicate titrations. The S.E. Est. proved to be 0.38. Since the slope of the line of best fit is so close to 1.0, it would be expected that the value of a second determination would be approximately equal to the value of the first except for the influence of errors due to chance. Hence a difference between any 2 end point determinations greater than twice the S.E. Est. ($2 \times 0.38 = 0.76$ or .8) may be considered significant at the 95% level of confidence and a difference 3 times that value, or 1.1, significant at the 99.7% level. These limits are indicated in Fig. 1. Attention is called to the similarity of the value of the difference between any 2 titrations required for significance, as derived in this and in the preceding section. This is not surprising since the assumptions underlying the 2 methods are the same.

Comparison of the variation of end point determinations with other strains of influenza virus and with other methods of titration. In Table II a summary is presented of the difference required for significance in end point determinations employing influenza viruses, as described in this and in previous reports(1,2). The data concerning titrations of purified suspensions of the PR8 strain of influenza virus in mice and in eggs have been recalculated, so that probability is expressed in per cent. The differences between successive end point determinations required for significance at various levels of confidence are remarkably similar even though the data were compiled from the results of different investigators employing different suspensions of influenza viruses in titration of infectivity in eggs or in mice. These observations suggest that in general when standardized techniques are followed, the reliability of EID$_{50}$ determinations is a function of errors inherent in these methods. The reliability appears to be independent of the strain of virus used, the source of virus suspension employed, or the host used for infectivity titration.

Summary. 1. The use of 2 different methods for analyzing the reliability of 50% end point determination has been illustrated. The data were available because duplicate titrations of a number of virus suspensions were required for the purposes of other experiments. Data of comparable significance could have been obtained by a similar number of replicate titrations of the same virus suspension. The latter type of procedure is tedious, expensive, and is seldom carried out. It would seem likely that the records of other investigators would contain data suitable for analysis by the methods used in this report. 2. In the present study the reliability of 50% egg infectivity titrations was determined with crude suspensions of lungs infected with recently isolated strains of Type A-prime and Type B influenza viruses. The reliability of these determinations was similar to that previously described for titrations of infectivity for mice or for eggs with purified suspensions of the PR8 strain of influenza virus.

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Effect of Acetoacetate and β-Hydroxy Butyrate on Riboflavin and Nicotinic Acid. (20003)

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It has recently been reported(1) that sodium acetoacetate on prolonged injection in rabbits brings about several diabetic symptoms associated with increase of blood sugar, decrease of glucose tolerance and depletion of glycogen storage in liver and muscle. This substance has also been found to cause depletion of plasma ascorbic acid and increase of blood lactic acid(2), disturbance in the balance of hepatic glycogenesis and glycogenolysis(3) and increase in blood urea and inorganic phosphate(4). It has also been shown(5) that acetoacetate and β-hydroxy butyrate, when injected intraperitoneally for a considerable period cause enormous depletion of vit. B_1 in blood and urine thus accounting for the observed neuritic conditions in the old cases of diabetic patients. Besides development of neuritic conditions through deficiency of vit. B_1, other symptoms such as glossitis and stomatitis are also frequently noticed in diabetics suffering for a long period, which have been found by Sydenstricker *et al.* (6) to be cured by nicotinic acid. Several investigators showed how members of the vit. B_2 complex have a beneficial role in carbohydrate metabolism. Gordon(7) noticed a drop of blood sugar by oral administration of nicotinic acid amide in diabetic patients and Ricciute(8) reported its importance in raising the low glycogen content of muscle and liver. Schroeder(9) has shown that riboflavin is helpful in relieving hyperglycemia and according to the findings of Collazo and Bayo(10) the biochemical functions of riboflavin consist in promoting the accumulation of liver and muscle glycogen from administered glucose or lactic acid produced in the organism.

Since carbohydrate metabolism is disturbed by the ketone bodies and since nicotinic acid amide and riboflavin are the constituent parts of coenzymes I and II and the yellow enzyme, the presence of which is essential for proper utilization of carbohydrate, it was thought worthwhile to ascertain whether the substances like acetoacetate and β-hydroxy butyrate have any destructive influence on these vitamins *in vivo*. The present paper will show how both the substances on injection to rabbits cause tremendous depletion of nicotinic acid as well as riboflavin in blood and urine.

Experimental. Twenty healthy rabbits each weighing approximately 2 kg were selected. They were kept on a diet of germinated gram (*Cicer arietinum*). They were divided into 3 groups of 8, 8, and 4. Group 1 was given daily injection of sodium acetoacetate. Group 2 was injected with sodium β-hydroxy butyrate intraperitoneally in doses shown in Table I. The third group was not given any injection (control). For riboflavin estimation in blood, the blood sample was first deproteinized by trichloroacetic acid and kept overnight in the incubator. After incubation, the riboflavin contents of the blood were estimated according to the method of Burch *et al.*(11). Urine riboflavin was estimated according to

TABLE I. Blood and Urinary Riboflavin and Nicotinic Acid Contents of Rabbits Receiving Repeated Daily Injection of Sodium Acetoacetate and β-hydroxy Butyrate.

No. of rabbits	Substance inj.	Days of inj.	Blood content of Riboflavin (γ/100 cc)	Blood content of Nicotinic acid (mg/100 cc)	Daily urinary excretion of Riboflavin (γ)	Daily urinary excretion of Nicotinic acid (mg)
8	Acetoacetate*	0	39.5 ± 6.55	.71 ± .05	40.1 ± 4.80	1.9 ± .33
8		15	31.8 ± 4.42	.61 ± .04	38.5 ± 4.66	—
7		40	20.6 ± 1.97	.47 ± .06	16 ± 3.91	.36 ± .05
5		90	14.3 ± 2.77	.13 ± .04	9.2 ± 1.99	.05 ± .03
8	β-hydroxy butyrate†	0	41 ± 2.74	.72 ± .09	40.7 ± 3.69	2.1 ± .18
8		15	20.4 ± 5.31	.63 ± .06	39.6 ± 3.57	—
8		40	21.5 ± 6.84	.48 ± .06	19.7 ± 5.25	.44 ± .08
8		90	9 ± 1.88	.18 ± .04	10.9 ± 4.26	.04 ± .03
4	Control	0	44.2 ± 3.05	.71 ± .02	45.4 ± 2.90	2.60 ± .33
4		15	41.9 ± 2.78	.71 ± .05	48.9 ± 4.11	—
4		40	43.8 ± 3.08	.70 ± .07	43.5 ± 2.17	2.62 ± .22
4		90	45 ± 3.91	.73 ± .03	41.6 ± .93	2.63 ± .26

* Daily dose of acetoacetate inj.: 1st to 15th day—100 mg/kg; 16th to 30th day—150 mg/kg; 31st to 45th day—200 mg/kg; 46th to 60th day—250 mg/kg; 61st to 75th day—300 mg/kg; 76th to 90th day—350 mg/kg.

† Daily dose of β-hydroxy butyrate: 1st to 15th day—100 mg/kg; 16th to 30th day—125 mg/kg; 31st to 45th day—150 mg/kg; 46th to 60th day—175 mg/kg; 61st to 75th day—200 mg/kg; 76th to 90th day—250 mg/kg.

the method of Ferrebee(12). For the estimation of nicotinic acid in blood the method of Swaminathan(13) was used, while the method of Wang and Kodick(14) was used for determination of nicotinic acid in urine.

Results. It is evident from Table I that rabbits receiving sodium acetoacetate and β-hydroxy butyrate injection for 90 days show a more marked depletion of riboflavin and nicotinic acid in blood than in control animals. The daily excretion of these vitamins in urine is lowered considerably during the experimental period. About 75% of the rabbits injected with sodium β-hydroxy butyrate show severe lesions over the nose, face, legs and paws, and 50% of the animals injected with sodium acetoacetate show this type of skin lesion.

Discussions. Animals injected with sodium salts of acetoacetic acid and β-hydroxybutyric acid show depletion of riboflavin and nicotinic acid in blood. The daily excretion of these vitamins in urine is also lowered. The depletion may be either due to the destruction of these vitamins due to excess accumulation in the system of the ketone bodies or due to the excess demand for the coenzymes containing riboflavin and nicotinic acid as the prosthetic groups required for the metabolism of the accumulated acetone bodies. It is believed that the mechanism of hydrogen transport to oxygen in the Krebs' cycle is helped by di- and tri-phosphopyridine nucleotides (coenzymes I and II) and flavoproteins and cytochromes. That acetoacetate is metabolized through the Krebs' cycle was postulated by Breusch(15), and Wieland and Rosenthal(16) and confirmed by Buchman *et al.*(17). Unless these vitamins are given sufficiently along with the food, it is likely that the system will soon exhaust all these vitamin reserves or those little amounts served with the diets and will ultimately suffer from their deficiencies. It has actually been observed by Mannering, Lipton, and Elvehjem(18) that symptoms of riboflavin deficiency in rats increase in severity upon administration of increased amounts of fats. They have further observed that when increased doses of riboflavin are given together with increased amounts of fats, no deleterious effect is observed. Our present finding will also serve to explain how proper utilization of lactic acid (which requires the help of coenzyme I and yellow enzyme) does not take place in animals treated with acetoacetate and accumulates in the system, as has been reported earlier(2).

Summary. 1. Rabbits receiving continuous injection of sodium acetoacetate and β-hydroxy butyrate for 3 months show a state of riboflavin and nicotinic acid deficiency. 2. Both nicotinic acid and riboflavin levels in

blood of these animals are lowered considerably after 90 days of injection. 3. Marked decrease in daily excretion of these vitamins in urine has also been observed. 4. Animals show severe skin lesions over the nose, face, paws and legs.

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Effects of Proteins on Body and Organ Weights in Thyroid-Fed Rats.

(20004)

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We recently reported the effects of defatted liver residue, Liver Concentrate N.F. and Vit. B_{12} on body and organ weights of thyroid fed rats(1). This confirmed the data of Ershoff (2,3), Betheil *et al.*(4,5), and Emerson and Folkers(6,7) that liver residue contains an "anti-thyrotoxic factor."

We have extended this study to include soybean alpha protein and the partially purified animal and vegetable proteins distributed by the Rutgers University Bureau of Biological Research.

Procedure. The basal diet and animals employed were identical to those previously reported(1). The protein supplements were added in isonitrogenous amounts. The following proteins were studied: *Casein (1.12; 13.66),* beef muscle (4.68; 14.92), egg albumin (7.03; 12.59), wheat gluten (0.67; 13.38), peanut flour (4.18; 9.61) and whole egg (5.1; 11.85).* These proteins were obtained from the Rutgers University Bureau of Biological Research. They have been the subject of many studies, and methods of their preparation have been published(8). *Alpha protein (1.52; 13.8),* obtained from the Glidden Co., Chicago, Ill. This was supplemented with 2% d,l-methionine prior to use. *Defatted liver residue (2.61; 13.92)* similar to that used in our previous publication(1).

Results and discussion. Our data are reported in Table I. As previously shown, defatted liver residue counteracts the growth retardation due to thyroid and permits maintenance of normal adrenal and thymus weights.

Since defatted liver residue is largely protein, it was of interest to examine other proteins as a source of "anti-thyrotoxic factor". Our results show that when tested by their

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* First figure in parenthesis is % ash. Second figure % total nitrogen.

TABLE I. Effect of Liver Residue, Rutgers Proteins and Alpha Protein on Body and Organ Weights of Thyroid-Fed Rats.* (The experiment continued for 28 days; 8 rats started per group.)

Dietary supplement, %	Thyroid, %	No. surviving rats	Gain body wt, g (mean ± S.E.)‡	Adrenal wt, mg† (mean ± S.E.)‡	Adrenal wt,† mg /100 g body wt	Thymus wt, mg (mean ± S.E.)‡	Thymus wt, mg /100 g body wt
0	.0	8	101 ± 7.6	25.4 ± 1.29	18.6	436 ± 30	320
0	.2	6	74 ± 5.3	50.4 ± .88	46.5	213 ± 29	197
Defatted liver residue, 10	.2	7	99 ± 4.6	35.2 ± 1.37	26.2	344 ± 30	256
Casein, 10.2	.2	5	77 ± 3.4	39.2 ± .95	35.5	284 ± 31	256
Beef muscle, 9.3	.2	7	87 ± 5.6	30.6 ± 1.03	25.3	272 ± 23	224
Egg albumin, 11	.2	6	82 ± 7.4	47.8 ± 1.30	41	332 ± 11	284
Wheat gluten, 10.4	.2	7	88 ± 2.2	50.4 ± 1.52	40.5	293 ± 29	237
Peanut flour, 14.5	.2	3	96 ± 7.2	63.2 ± 1.81	48.3	336 ± 30	260
Whole egg, 11.7	.2	8	93 ± 2.6	60.8 ± 1.36	47.3	446 ± 42	350
Alpha protein,§ 10	.2	5	78 ± 7	49.6 ± 1.01	44.2	382 ± 24	340

* In one experiment a dietary supplement of 10% Wilson's edible porcine gelatin did not prevent weight loss in thyroid fed rats.

† In each case the figures represent the weight of both adrenals.

‡ Stand. dev. of mean = $\sqrt{\frac{\Sigma d^2}{(n)\ (n-1)}}$ where "d" is the variation of the individual measurement from the mean and "n" is the number of measurements.

§ Alpha protein was supplemented with 2% d,l-methionine prior to use.

effect on growth and survival, proteins vary in their content of this factor. Casein and alpha protein are very poor sources. Beef muscle, egg albumin and wheat gluten are better sources, while whole egg is comparable to defatted liver residue in its effect on growth and survival. Peanut flower may also contain the factor, but survival is poor in our experiments.

Those proteins with an anti-thyrotoxic effect similar to liver residue, as measured by growth and survival, are not necessarily similar in their effects on organ weights. Egg albumin, peanut flower and whole egg, none of which affect the adrenal, maintain thymus weight. Alpha protein, when supplemented with 2% d,l-methionine, has an amino acid pattern equal to some of the best proteins, and yet gives poor performance for growth and the maintenance of normal adrenal weight. It is effective in maintenance of thymus weight. Beef muscle is the only protein tested which resembles defatted liver residue in all the anti-thyrotoxic effects measured.

More than one factor is necessary to counteract the effects of thyroid in the diet, since some proteins prevent growth inhibition but do not affect organ weight changes. Conversely, other proteins maintain normal organ weights but do not counteract growth inhibition. Whether these differences are due to unknown factors or to a proper balance of those already known, cannot be stated at this time. Since more than one factor is involved, it is possible that a mixture of several proteins would be more efficient than a single protein in counteracting all the effects of thyroid in the diet of the rat.

Summary. 1. Several proteins have been compared to defatted liver residue in their effects on body and organ weights of thyroid fed rats. 2. Beef muscle is the only protein studied which gives results similar to defatted liver residue. 3. Several factors are involved, since some proteins maintain body weight but do not affect organ weight. Other proteins affect organ weights, but do not maintain body weight in the thyroid fed rat.

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Effect of Pentobarbital Sodium Anesthesia upon Volume Blood Flow, Arterial Pressure and Heart Rate.* (20005)

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Pentobarbital sodium is one of the most widely used general anesthetics for animal experimentation. Few studies have been reported concerning the effects of this barbiturate upon peripheral circulation. Richter and Oughterson(1) reported that its administration was followed by a marked vasodilator effect. Tabern(2) found that this drug had little effect on blood pressure and respiration when administered to laboratory animals in doses of 30 to 45 mg per kg of body weight. Morrison, Walker, and Richardson(3) found that gradually increasing doses of pentobarbital produced a gradual fall in blood pressure and a marked increase in pulse rate of dogs. The experiments described in this report are concerned with the effects of anesthetic doses of pentobarbital sodium upon pulse rate, arterial pressure and volume of blood flow through the femoral artery of adult dogs.

Method. The methods employed in these studies permitted the continuous recording of volume blood flow, arterial pressure and heart rate before, during and after the intravenous injection of an anesthetic dose of pentobarbital sodium (35 mg per kg of body weight). Volume blood flow was measured through the femoral artery by means of an electromagnetic flow meter with the aid of heparin as an anticoagulant(4). Pre-anesthetic control measurements were made on trained animals. This procedure required infiltration of the area of cannulation with a 2% solution of procaine hydrochloride. A preliminary period lasting from 15 to 30 minutes was allowed for recording pre-anesthetic values, after which time anesthesia was induced by intravenous injection of 35 mg of pentobarbital sodium per kg of body weight. Injection time required approximately 30 seconds. The anesthetic was made up as a 7% solution of pentobarbital sodium in 15% ethanol.

Results. The results from experiments on 8 dogs are summarized in Table I. Administration of anesthetic doses of pentobarbital sodium was followed by a transient increase in the volume blood flow through the femoral artery, which usually returned to pre-anesthetic values within 15 minutes. In a few cases the increase in blood flow persisted for as long as 30 minutes. Mean arterial pressure declined after anesthetic administration and gradually returned to approximately pre-anesthesia values within 15 to 30 minutes. Increased pulse rate was noted throughout the duration of anesthesia. The administration of an amount of ethanol which would be present in an anesthetic dose of a solution of pentobarbital was found not to cause significant changes in arterial pressure and volume blood flow. In other experiments it was found that the administration of "booster" doses of pentobarbital sodium in amounts of 10 to 20 mg per kg of body weight was followed by transient changes in arterial pressure and femoral artery blood flow similar to those found for full anesthetic doses of the drug.

The results of this investigation indicate

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* Aided by a grant from the Central Scientific Fund of the College of Medicine, State University of Iowa.

TABLE I. Effect of Pentobarbital Sodium Anesthesia upon Volume Blood Flow, Mean Arterial Pressure and Pulse Rate.

	Pre-anesthetic control	% change from control, min. after inj.				
		.5	1	2	5	15
Flow in cc/min.	45	+119	+110	+62	+13	0
S.D.		97	88	29	25	—
S.L.*		1%	1%	1%	18%	—
Pressure, mm/Hg	130	—16	—35	—33	—17	—12
S.D.		11	6	17	14	10
S.L.*		1%	1%	1%	2%	3%
Pulse rate/min.	110	+34	+36	+41	+39	+39
S.D.		25	20	26	25	25
S.L.*		1%	1%	1%	1%	1%

* Level of significance.

the necessity of being cognizant of the effect of pentobarbital sodium anesthesia *per se* upon volume blood flow and arterial pressure in any experiments in which measurement of changes in these factors are pertinent to a study. Attention should also be called to the finding that supplemental administration of the drug for the purpose of maintaining satisfactory surgical anesthesia was also followed by transient changes of a significant magnitude in blood flow and arterial pressure. A sufficient period of time should be allowed to elapse following anesthetic administration either for recovery of blood pressure and flow to pre-anesthetic control values or to a new steady state. Our findings indicate that considerable variation exists in the time required for such readjustments to be made in experiments on different animals.

Summary. A study was made with the aid of continuous recording of the effects of an anesthetic dose of pentobarbital sodium and of supplemental doses of this drug required for the maintenance of surgical anesthesia upon arterial pressure, pulse rate and volume blood flow through the femoral artery of dogs. Following the administration of this anesthetic there was an immediate increase in volume blood flow and a fall in mean arterial pressure. Recovery to near pre-anesthetic control values usually occurred within 5 to 15 minutes. However, in some experiments recovery was not apparent for as long as 30 minutes after drug administration. Pulse rate remained increased throughout the duration of anesthesia.

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Adrenals and Anterior Hypophysis in Leukemogenesis of Mice.* (20006)

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In male castrate mice of strain A, anterior hypophyseal transplants significantly increased the incidence of malignant lymphoma (1). This result was tentatively explained as due to hyperadrenalism caused by the grafted hypophyses: Hyperadrenalism leads to depletion of lymphoid tissue, and protracted atrophy of the latter may be followed by hyperplasia and eventually neoplasia(2-4). On the

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* Aided by a Grant from the Brant Fund of the Am. Med. Assn.

TABLE I. Incidence of Leukemia and Leukemia Age.

Series	No. of mice Total	Of leukemia age	With leukemia	% of leukemia	Leukemia age, mo Mean	Range
Castrates	39	32	2	6.3	17.5	13-22
Castrates bearing adrenal grafts	38	30	4	13.3	12.8	10-18
Castrates bearing adrenal and hypophyseal grafts	40	32	8	25	12.5	8-19
Castrates bearing (1) hypophyseal grafts	28	28	7	25	16.6	9-20

other hand, in adrenalectomized rats adrenal transplants inhibited growth of transplanted lymphatic leukemia(5); while, likewise in rats, lymphosarcoma was observed following treatment with anterior hypophyseal growth hormone(6). In order to further test the role of adrenals and hypophyses in leukemogenesis, the following experiments were carried out.

Material and methods. One hundred and twenty newborn mice of the closely inbred strain A, raised in our laboratory, were orchidectomized and after weaning divided into 3 groups of 40 animals each. *Series I:* The castrates received no further treatment. *Series II:* The castrates received subcutaneous transplants of 4 adrenals; 8 mice received adrenals from male, 27 from female, and 5 from both male and female donors. *Series III:* The castrates received 4 adrenals and 4 hypophyses; 10 mice received adrenals from male, 28 from female, and 2 from both male and female donors; 7 mice received hypophyses from male, 25 from female, and 8 from both male and female donors. All grafts were obtained from 1- to 3-month-old donors closely related to the recipient. The animals were fed a stock diet of Purina Laboratory Chow and water *ad libitum.* Two mice of each series were sacrificed at the ages of 2, 4, 5, or 7 months; later the animals were killed as their condition warranted it. Some mice died unexpectedly, and in a few of these autolysis was too far advanced to permit microscopic examination of the tissues. These were excluded from the investigation. Details may be seen from the table. At autopsy, pieces of internal organs, the legs, mammary glands, thyroids, hypophyses, adrenals and transplants, when present, were removed, fixed in Bouin's solution and embedded in paraffin. Semiserial sections stained with hematoxylin and eosin were prepared for microscopic studies. Some hypophyses and hypophyseal grafts were fixed in Regaud's solution for cytological and histochemical studies.

Results. Only those cases were classified as leukemia that could be diagnosed grossly and that microscopically showed extensive infiltrations of lungs, liver, spleen, heart and kidneys.

Series I: Among 39 castrates 32 lived longer than 8 months. This was the earliest age at which leukemia was observed in any of our animals. Of these mice only two (6.3%) had lymphatic leukemia at the mean age of 17.5 months, as compared with a 3.6% incidence at a mean age of 16.9 months in our controls. *Series II:* Of 38 castrates bearing adrenal grafts 30 reached leukemia age, and of these 13.3% had lymphatic leukemia at a mean age of 12.8 months. In 20 of these mice (66.7%) living adrenal grafts were found microscopically. *Series III:* Of 40 castrates carrying adrenal and hypophyseal grafts 32 reached leukemia age, and of these 25% had developed lymphatic leukemia at a mean age of 12.5 months. In 19 of these mice (59.4%) adrenal and in 17 mice (53.1%) hypophyseal grafts were identified microscopically.†

Fate of grafts in leukemic mice. Grafts were found in 3 of 4 mice bearing adrenal transplants (Fig. 1, 2). Some grafts underwent hyperplastic changes similar to those

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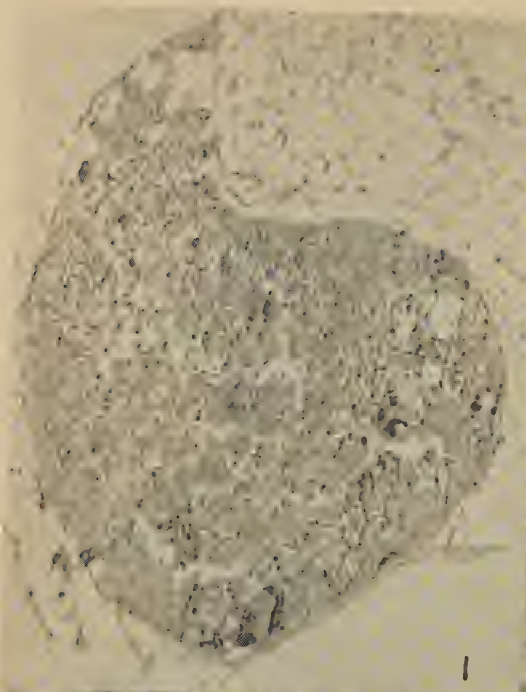
† The actual number of mice showing hypophyseal grafts was probably higher, since tissue grossly most suggestive of "takes" was retained for cytological studies which will be reported at a later date.



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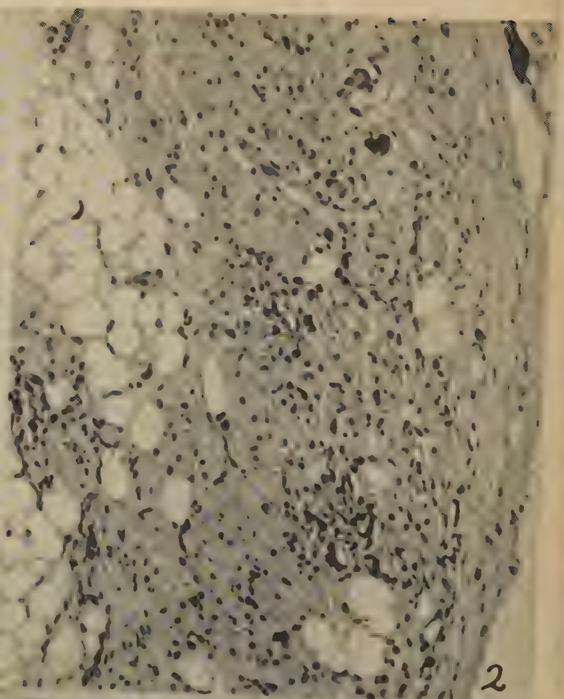

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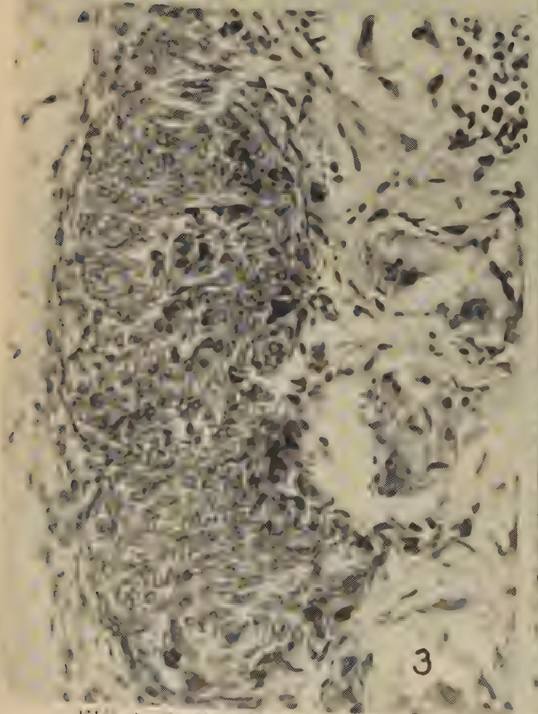

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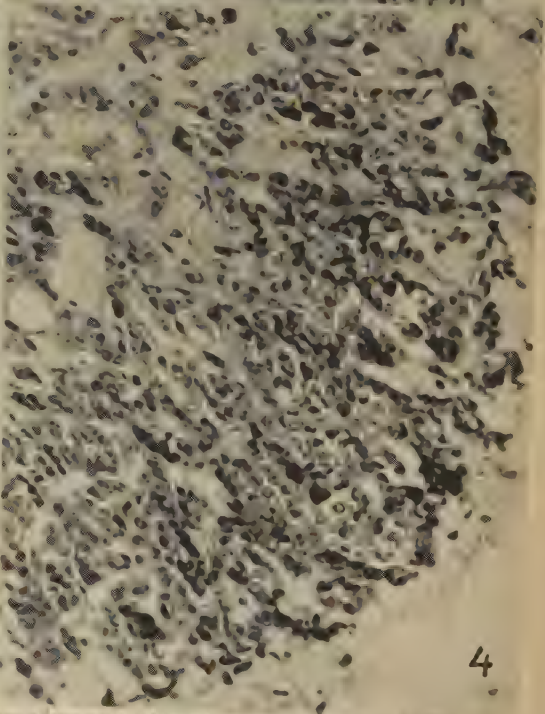

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FIG. 1. Section through an adrenal graft 10 mo after transplantation. Magnification × 75.

FIG. 2. Section through an adrenal graft 10 mo after transplantation. The mouse has also received hypophyseal grafts. Magnification × 290.

FIG. 3. Section through another area of the transplant shown in Fig. 2 demonstrating a hyperplastic focus in the adrenal cortex. Magnification × 300.

FIG. 4. Section through a hypophyseal graft 18 mo after transplantation. Magnification × 300.

seen in the animals' own adrenals (Fig. 3). Of 8 leukemic mice carrying adrenal and hypophyseal grafts, adrenal tissue was recovered in 6 and hypophyseal grafts in 5 animals (Fig. 4). Obviously, there was no difference in the stimulating effects of male or female grafted glands.

Structure of adrenals in leukemic animals. Of the 2 untreated leukemic castrates one showed slight focal cortical hyperplasia and the other one cortical adenomas. Of the 4 leukemic castrates bearing adrenal grafts, one showed resting adrenal cortex, two slight focal cortical hyperplasia and one cortical adenomas. Of the 8 leukemic castrates bearing adrenal and hypophyseal grafts, one showed resting adrenal cortex, one slight focal, and one diffuse cortical hyperplasia, while the remaining 5 animals showed cortical adenomas.

Structure of mammary glands in leukemic animals. In order to determine whether or not the adrenal changes were associated with the production of estrogen, the mammary glands were investigated. In none of the leukemic mice was acinar growth stimulation present. Of the total of 14 leukemic mice, 10 had resting mammary glands, and only 4 showed slight ductal growth stimulation. Therefore, any estrogenic effect if present at all would have been slight at best and could hardly account for leukemogenesis.

Discussion. Orchidectomy performed in newborn mice of strain A increased the incidence of leukemia from 3.5% in untreated controls to 6.3%. In castrates bearing adrenal transplants the incidence of leukemia was further increased to 13.3%, and the mean age of the animals at the time of leukemia development was advanced by 4.7 months as compared with that seen in untreated castrates. This finding which represents a considerable shortening of the latent period may be attributed to the action of the grafted adrenals. As reported previously, in mice of strain A orchidectomized at the age of one month and bearing only hypophyseal transplants, a 25% incidence of leukemia was observed at a mean age of 16.6 months(1). In newborn castrates carrying adrenal and hypophyseal transplants, the incidence of leukemia was likewise 25%. However, the induction period was 4.1 months shorter than in castrates carrying hypophyseal grafts only. This finding again points to the role of the adrenal grafts in shortening the latent period of leukemia. None of the leukemic animals showed acinar growth of the mammary gland, and none of the non-leukemic animals developed mammary cancer, although some of the latter mice showed acinar hyperplasia. Therefore, the adrenal cortex did not produce large amounts of estrogen, and thus estrogen might not have played the primary role in this type of leukemogenesis. On the other hand, the present results do not rule out a direct effect of the hypophysis on hemopoietic tissues; however, the adrenal cortex seems to take part in the evolution of the leukemic process.

Summary. In newborn mice of strain A, orchidectomy slight increased the incidence of leukemia. In such castrates, adrenal transplants doubled the incidence and conspicuously advanced the onset of the disease. Hypophyseal transplants made in conjunction with adrenal grafts increased the incidence of leukemia 4 times over that seen in non-grafted castrates. Prolonged hyperpituitarism associated with hyperadrenalism apparently exerted this leukemogenic effect.

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Effect of ACTH Administration on Certain Enzyme Systems in Rat Liver.* (20007)

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According to Russell and Wilhelmi(1), Jensen and Gray(2), and Umbreit and Tonhazy(3), adrenal cortical hormones affect the activity of D-amino acid oxidase. The proline oxidase of kidney(3) and arginase(4) are also influenced by the hormones. With the exception of a study on creatine synthesis by Umbreit and Tonhazy(3) in which they attributed their results to the effect of cortisone on the oxidation of D-methionine to the keto analog, no work has been reported on the effect of increased cortical activity on other energy requiring systems, including other methylations. The following is a report on the effect of ACTH on several such systems in rat liver including the effect on fatty acid oxidation.

Experimental. 1.0 mg ACTH (Corticotropin, Wilson) was injected daily into male rats weighing 150-300 g for 7-10 days. They, and a control of the same weight, were killed by a blow on the head, and subsequent bleeding. Liver slices were incubated in a Dubnoff shaker at 37°C in Krebs-bicarbonate solution and 95% O_2-5% CO_2 gas mixture. The end products of the methylation of nicotinamide and guanidoacetic by methionine and the formation of methionine itself from homocysteine and betaine were estimated according to the methods described in the original papers(5-7). Urea was estimated according to the method of Barker(8), tyrosine from phenylalanine by the method of Theis and Benedict(9) and uric acid from xanthine by the method of Folin (10).

The production of acetoacetate from octanoate was determined in 3 groups of rats by the method of Greenberg and Lester(11). Slices were incubated from animals which (A) had been fasted for 72 hours and then injected with 1.0 mg ACTH hourly for 3 hours before killing; (B) fed *ad libitum* and injected in the same way; and (C) fed *ad libitum* and injected with 1.0 mg ACTH daily for 8 days Table I shows the effect of ACTH administration for 7-10 days on the methylation of 3 compounds. The methylation of nicotinamide was inhibited, that of homocysteine unaffected. and that of guanidoacetic acid increased. L-methionine was used and the color given by the keto acid when methionine was incubated alone was subtracted from that given by methionine plus creatine formed from the guanidoacetic acid. This differential effect of ACTH on the 3 methylations indicates that separate systems are involved.

Table I shows that the conversion of phenylalanine to tyrosine was depressed in the livers of ACTH-treated animals. Uric acid production from xanthine anaerobically with pyruvate as the hydrogen acceptor(12) was somewhat decreased whereas urea production from ammonium sulfate or glutamine was not affected.

Table I also shows that endogenous acetoacetate production as well as the oxidation of added octanoate was increased by ACTH injected for 7-10 days. The acute effects of ACTH were only seen when the rats were starved for 72 hours. These results on the fatty acid oxidase activity are in agreement with those of Shipley(13), Campbell and Davison(14) on the ketogenic effect of pituitary extracts, of Lipsett and Moore(15) on the effect of cortisone, and the *in vivo* results of Bennett *et al.*(16) on the ketonemia produced by ACTH and by growth hormone. They are in disagreement with the experiments of Wilhelmi and Bondy(17) on the effect of ACTH and growth hormone on endogenous ketone production by liver slices.

Discussion. With the exception of group A animals in the experiments on ketone production, the effects of ACTH were only evident after a relatively prolonged treatment.

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TABLE I. Effect of ACTH on Certain Reactions in Rat Liver. 250 mg (wet wt) of slices were incubated 3 hr at 37°. Final concentrations of substrates were as follows: 2×10^{-4} M nicotinamide (NA), 3.3×10^{-4} M l-methionine (M), 1×10^{-3} M homocysteine (HC), 1.1×10^{-2} M betaine (B), 5×10^{-4} M guanidoacetic acid (GAA), 3.03×10^{-3} M l-phenylalanine (PA), and 7.3×10^{-3} M Na octanoate (OCT).

	Control				After ACTH				P significant at:	
Reaction	No. of animals				No. of animals				1%	.01%
Methyl nicotinamide from NA and M as μg/g wet wt		NA	NA+M			NA	NA+M			
	5	12.8	33.4		8	3.3	12.1		+	±
Methionine from HC and B as mg/g wet wt		HC	HC+B			HC	HC+B			
	9	.24	1.01		7	.23	1.01		—	—
Creatine from GAA and M as μg/g wet wt		M	M+GAA	Diff.		M	M+GAA	Diff.		
	7	55	112	57	7	49.7	157.2	107.5	+	±
Tyrosine from PA as mg/g wet wt		No add.	PA			No add.	PA			
	8	1.18	3.86		8	.79	2.04		+	+
Acetoacetate from OCT as μg acetone per g wet wt		No add.	OCT	Diff.		No add.	OCT	Diff.		
Treatment A	7	502	1239	737	7	927	2101	1174	+	+
B	4	314	731	417	4	511	726	215	—	—
C	3	231	748	517	4	433	1516	1077	+	+

Since this brings about changes in liver glycogen, fat and minerals, the hormone effect may be the result of these changes rather than a direct action on the enzyme systems. It is also possible that growth hormone (or another pituitary factor) which may be a contaminant in the preparation may be responsible for some of the results. Engel(18) has found that ketosis following ACTH administration is not primarily the result of stimulation of the adrenal gland, and Tepperman and Tepperman(19) showed that pretreatment of hypophysectomized rats with cortisone caused ketosis in liver slices when growth hormone was administered. In group A, fasting may be considered a stress, which might have conditioned the animal to the action of the "ketogenic" pituitary factor.

Summary. 1.0 mg ACTH was injected into rats for 8-10 days. Liver slices from these animals showed a decreased methylation of nicotinamide and an increased methylation of guanidoacetic acid. The methylation of homocysteine was unaffected. Tyrosine formation from phenylalanine was inhibited as was uric acid formation from xanthine. Urea production was normal. Endogenous ketone body production and the oxidation of octanoate was increased.

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Nutrition of the Mouse. XII. Comparison of Several Salt Mixtures.* (20008)

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Although a wide variety of salt mixtures, many of these commercially available, is in use in nutritional experiments with mice, there is at the present time little information about the relative merits of these preparations. For this reason we decided to compare five salt mixtures fed at a 5% level as part of an otherwise complete synthetic diet.

Method. Weanling mice of the C57 and I strains were divided into 5 comparable groups, each receiving the same basal diet (Table I) but a different salt mixture. The following preparations were employed: Hubbell, Mendel and Wakeman(1), Wesson(2), U.S.P. XII No. 2, McCollum and Simmonds No. 185(3) and Fenton and Carr(4). The mice were housed in individual screen-bottom metal cages in a constant temperature room. Food and water were supplied *ad libitum.* The animals were weighed once each week and sacrificed when 135 days of age. At this time determinations of water, fat and ash content were carried out.

TABLE I. Composition of Diet in %.*

Casein	30	Corn oil	5
Dextrin	50	Salt mixture	5
Crisco	10		

* Vitamins added in amounts described by Fenton and Carr(4).

Results and discussion. The experimental results are summarized in Table II. Although variations among groups were encountered with respect to final body weight and total fat content, no striking effects of varying the salt mixture were seen. With each salt mixture employed there was, however, considerable difference between the 2 strains of mice with respect to final body weight and total carcass fat. The dry, fat-free weight of all groups of mice was virtually the same. Plotting the body fat content against percent body water showed the inverse linear relationship already reported by other investigators. The ash content of mice fed the diet containing Hubbell, Mendel and Wakeman salts was significantly higher than the ash content of any other group. This was true for both strains of mice. The values obtained for total ash content are in good agreement with those of Morris(5).

The most striking difference between the salt mixture described by Hubbell, Mendel and Wakeman and that described by Fenton and Carr lies in the Ca:P ratio. This ratio is close to unity in the Fenton and Carr mixture but is considerably higher in the Hubbell, Mendel and Wakeman preparation. For this reason a new salt mixture was prepared differing from the original Fenton and Carr preparation in that some of the calcium phosphate was replaced by calcium carbonate, thus retaining essentially the same calcium level but decreasing the phosphorus content. Two groups of 12 mice each were fed identical diets but one receiving the old while the other received the new salt preparation. The ash content of these mice when sacrificed at 135 days of age differed significantly, the new salt mixture giving a higher total body ash.

Summary. Five salt mixtures were tested for their ability to support growth as well as

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* Supported by grants from The Anna Fuller Fund and The American Cancer Society on recommendation of The Committee on Growth of The National Research Council.

TABLE II. Results of Carcass Analysis.

Salt mixture	Strain	No. of mice	Final body wt, g	Dry wt, g	Dry, fat-free wt, g	Fat, g	Ash, g
Hubbell, Mendel and Wakeman	C_{57}	6	31	13.4	6.2	7.2	.93 ± .016*
	I	5	29.6	10.7	6.5	4.3	.95 ± .015
Fenton and Carr	C_{57}	8	28.9	11.7	5.8	5.8	.86 ± .009
	I	5	26.4	8.3	5.8	2.5	.86 ± .015
Wesson	C_{57}	6	28	11	5.8	5.2	.85 ± .011
	I	5	26.9	9.1	5.8	3.5	.88 ± .021
U.S.P. XII #2	C_{57}	6	32.2	13.9	6.2	7.7	.84
	I	5	25.7	8.7	5.8	3	.86 ± .014
McCollum-Simmonds #185	C_{57}	6	29.8	12.4	6.1	6.3	.83 ± .007
	I	5	26.3	9.1	5.8	3.2	.84 ± .014

* Stand. error.

fat and mineral deposition in the carcass. Feeding the mixture described by Hubbell, Mendel and Wakeman(1) resulted in significantly higher ash values than when the other four mixtures were employed. Increasing the Ca:P ratio of the Fenton-Carr mixture(4) significantly improved mineral deposition.

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Cytoplasmic Budding of Human Lymphocytes Produced by Cortisone and Hydrocortisone in *in vitro* Preparations.* (20009)

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The destructive changes that lead to lymphocytic dissolution *in vivo* under conditions of increased adrenocortical secretions begin with cytoplasmic budding and result in pycnosis and karyorrhexis of the cells(1-3). Shedding of the cytoplasm of lymphocytes was described as a normal process in the older literature by Downey and Weidenreich and the cytoplasmic fragments were termed hyaline bodies(4). The observation of cytoplasmic budding of lymphocytes in this laboratory in blood films of patients treated with ACTH and cortisone led to an attempt to produce this phenomenon by treating lymphocytes isolated from buffy coat of normal human blood with cortisone and hydrocortisone *in vitro*.

Materials and Methods. Crystalline hydrocortisone (free alcohol) and cortisone (free alcohol) were used in these experiments. Ethylene bisiminodiacetic acid (Sequestrene Na_2) was used as an anti-coagulant according to the technique of Proescher(5). Fifteen ml samples of venous blood from normal human subjects were centrifuged in sterile tubes containing 0.1 ml of a 10% solution of Sequestrene Na_2. The hormone to be tested was added to a portion of plasma so that 1 ml of plasma contained 1 mg of hormone. Nonhormone treated plasma from the same sample was used for control studies. A drop of

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* These studies were aided by a grant from the Committee on Growth, National Research Council acting for the American Cancer Society.

† The authors wish to thank Dr. Augustus Gibson of Merck and Co. for the supply of hormone used and Dr. H. W. Zussman of the Alrose Chemical Co. for Sequestrene Na_2.

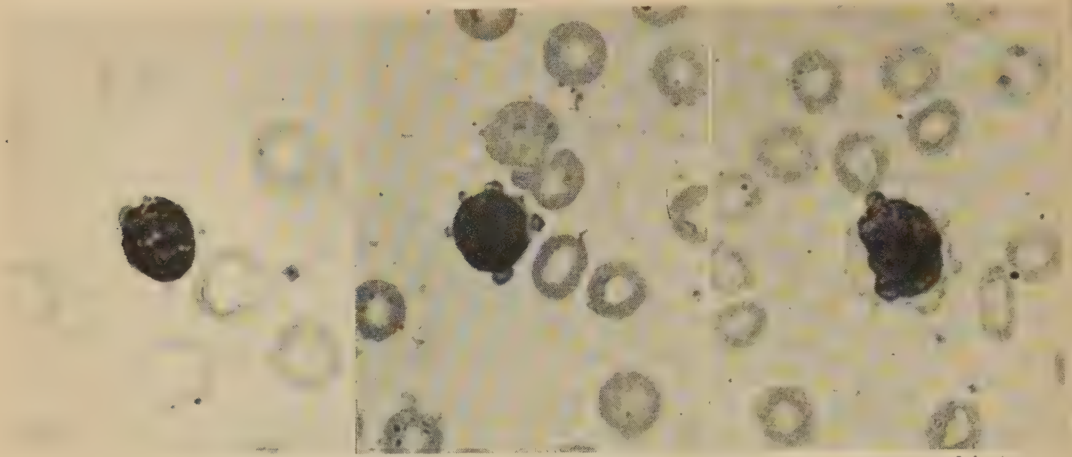

FIG. 1. Cytoplasmic budding in buffy coat lymphocytes obtained from normal human subjects and treated with hydrocortisone.

plasma was mixed gently with a drop of buffy coat from the same blood specimen on a clean cover slip and smeared. The mixing and smearing process occupied approximately 30 seconds. Cover slips were air dried and stained with May Grünwald Giemsa. A minimum of 200 lymphocytes per cover slip were counted.

Results. A comparison of the percent of budding lymphocytes in normal and treated buffy coat lymphocytes is seen in Table I. Cytoplasmic budding was found in 11.63% of the lymphocytes of the hydrocortisone treated buffy coat and in 1.2% of the controls. Cortisone also produced cytoplasmic budding but to a lesser degree than hydrocortisone. Buffy coat lymphocytes demonstrating cytoplasmic budding after treatment with hydrocortisone are presented in Fig. 1.

TABLE I. Percent of Budding Lymphocytes in Normal and Treated Buffy Coat Lymphocytes.

	Control	Experimental
No. of exp.	Buffy coat and plasma	Buffy coat, plasma and hydrocortisone
1	.25	10.5
2	0	10
3	1.25	9.75
4	0	7.5
5	0	8
6	2.5	20
7	4	18.25
8	1.5	8.75
9	0	15.75
10	2.5	7.75
$\bar{X}$	1.2	11.63
σm	.4	1.25

Discussion. Williamson recently confirmed the findings of Downey and Weidenreich(4) that lymphocytes from lymph and lymph nodes of rabbits, rats and guinea pigs shed their cytoplasm in *in vitro* preparations. He found that the medium sized lymphocytes were the most actively budding cells(6). Several authors concluded that lymphocytes contributed their cytoplasmic constituents to the body fluids by the apocrine type of secretion (2,4,6). Dougherty and White suggested that the phenomenon of lymphocytic cytoplasmic shedding is enhanced by increased concentrations of C-11-oxy adrenocortical steroids(2).

The production of cytoplasmic budding in circulating lymphocytes of hydrocortisone has been demonstrated. The free alcohol of hydrocortisone is soluble in plasma to the extent of 0.7 mg per milliliter(7). Therefore, it can be assumed that an excess of 0.3 mg per milliliter was present in suspension. No attempt was made to demonstrate the lymphocytolytic effects of adrenocortical hormones which are known to occur *in vivo*(2).

The problem as to whether or not the purified adrenocortical hormones exert lymphocytolytic effects *in vitro* is still not settled. Adrenal cortical extract (Lipo Adrenal-Upjohn) produced "bubbling" of lymphocyte cytoplasm and disintegration of lymphocytes (8). Hechter and Johnson however, found that lymphocytolysis occurred *in vitro* only when lymphatic tissue homogenates were added to the adrenal cortical extract and suggested that a co-factor present in the tissue was essential for lymphocytolysis(9). Other investigators were unable to confirm the lymphocytolytic action of adrenal cortical extracts using *in vitro* techniques(10,11).

Cortisone and hydrocortisone produce degeneration of lymphocytes *in vitro* as measured by degeneration and inhibition of cell migration in tissue culture(12) and eosin staining in lymphocyte suspensions(13). Purified adrenocortical steroid hormones which were not lymphocytolytic *in vivo* also were without effect *in vitro*(13).

It is apparent from the data presented here that cytoplasmic budding reflects an excess of the C-11-oxy, 17-hydroxy adrenocortical hormones at the lymphocyte level and is similar to the "bubbling" phenomenon described by Feldman following treatment with adrenal cortical extracts(8). It is suggested that lymphocytes that have undergone cytoplasmic budding may revert to normal lymphocytes. On the other hand, lymphocytes may undergo karyorrhexis and pycnosis. It is not known whether these various phenomena are due to variations in the concentration of adrenocortical hormones or whether there is a difference in susceptibility of lymphocytes. This problem is being investigated at the present time.

Summary. 1. Significant numbers of lymphocytes exhibiting cytoplasmic budding were found within 30 seconds following addition of cortisone or hydrocortisone to plasma and then to buffy coat of the blood of normal human subjects. It is suggested that cytoplasmic budding of lymphocytes may be a reversible process and that the occurrence of cytoplasmic budding, pycnosis or karyorrhexis may depend upon differential susceptibility of lymphocytes.

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Effects of Age, Food Intake, and Stress on Plasma Hexosamine Levels in the Rat. (20010)

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Non-specific body injury or stress has been shown to produce an increase in the level of plasma hexosamine in many acute and chronic clinical diseases(1-6) and under a variety of experimental conditions(7-10). In order to study the physiological mechanisms involved in this response it was first necessary to examine the effects of age, and food intake in the normal and stressed animal. In the rat these are shown to be critical variables calling for carefully controlled experimental conditions.

Methods. Male Sprague-Dawley rats were fed a specially prepared diet,* unless otherwise indicated, and drank water *ad libitum*. The rats were stabilized 3-7 days on this diet

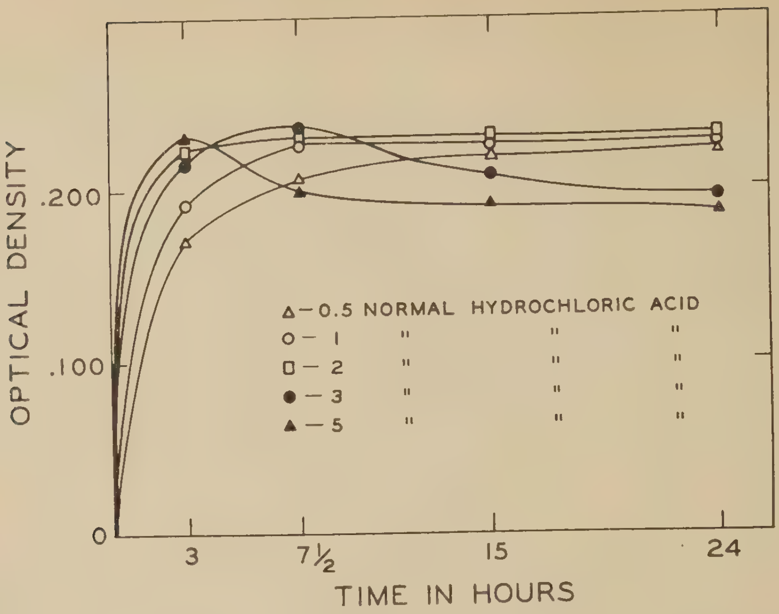

FIG. 1. Effect of acid hydrolysis (100°C) on rate of liberation and destruction of rat plasma hexosamine.

before experiments were started. Pair-fed controls, when used, were started one day later than the experimental groups. Food intake and body weights were measured daily. Tail blood samples (0.35 ml) were collected for the hexosamine determinations and external jugular vein blood for the plasma volume studies. All samples were treated with dry oxalate, transferred to Wintrobe hematocrit tubes and centrifuged at 3400 rpm for 30 minutes. Hematocrit readings were made and the plasma separated. For the hexosamine determinations 1.1 ml of water were added to 0.1 ml of each plasma sample. These diluted samples were stored in a refrigerator at 4°C until the end of an experiment,[†] when 1 ml aliquots were transferred to 10 ml glass-stoppered volumetric flasks. One ml of 4 N hydrochloric acid was added to each sample, producing a 2 N solution, the flasks were stoppered and placed in a boiling water bath for 15 hours. After cooling, they were taken to volume with water and filtered. The conditions for maximal liberation of hexosamine were established by hydrolyzing replicate plasma samples as indicated in Fig. 1. Two ml aliquots of the filtrates were used in a modified Elson and Morgan hexosamine method(12). Plasma chromogens interfering in the hexosamine method amount to less than 3-4% so no purification of plasma is necessary(12). For the 90-140 gram rats which were used, the hexosamine range varied generally from 120-140 mg/100 ml. In serial bleeding experiments the values are expressed as mg/100 ml change from an initial control value.

In experiments where 2 or more blood samples were obtained, the hematocrit values fell due to hemo-dilution resulting from blood loss. With fasting the hematocrit values increased as a result of dehydration. Both of these hematocrit changes were primarily the result of plasma volume changes. Since hexosamine is expressed as concentration in plasma, the hexosamine values in the fasting and serial bleeding experiments were therefore

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[*] Diet No. 4164(11): 67.5% ground whole wheat; 15% casein; 10% whole milk powder; 5.25% cotton seed oil; 1.5% $CaCO_3$; 0.75% NaCl; Vit. A 12,000 U.S.P. units, Vit. D 2,150 U.S.P. units and alpha-tocopherol 50 mg per kilo of diet respectively. (Protein 24.3%; carbohydrate 54.8%; fat 11.0%).

[†] Plasma hexosamine is stable at 4° for 2 weeks. For longer preservation the samples may be stored in a deep freeze.

TABLE I. Effect of Age on Hematocrit and Plasma Hexosamine Levels in the Rat.

Age (wk)	4	7	11	16
Body wt (g)	40 ± .9*	81 ± 1.5	159 ± 4.7	271 ± 10.6
Hematocrit (%)	44.8 ± .7	46.9 ± .8	51.7 ± .5	52.3 ± .2
Hexosamine (mg/100 ml)	94 ± 3.7	136 ± 6	162 ± 3.5	146 ± 1.3

* Stand. error of the mean.

TABLE II. Effect of Fasting on Blood Volume and Plasma Hexosamine.

	Control	Starved	t	P
No. rats	5	5		
Initial body wt (g)	132 ± 3.5 *	137 ± 4		
Final " " (g)	134 ± 4.9	107 ± 2.6		
Hematocrit (%)	43.7 ± 1.1	51.2 ± 1.7	7.42	$<.01$
Plasma Hexosamine (mg/100 ml)	119 ± 3.1	122 ± 2.9	1.41	$<.50$
Plasma volume (ml)	5.86 ± .24	4.47 ± .14	10.01	$<.01$
Red cell volume (ml)	5.02 ± .16	4.58 ± .16	3.89	$<.01$
Total blood volume (ml)	10.88 ± .19	9.05 ± .17	14	$<.01$
Total circulating hexosamine (mg)	6.96 ± .24	5.43 ± .16	10.60	$<.01$

* Stand. error of the mean.

TABLE III. Effect of Time of Sampling and Fasting on Plasma Hexosamine Levels in the Rat.

		1st day		2nd day	
		9 A.M.	5 P.M.	10 A.M.	5 P.M.
Control	Body wt (g)	130 ± 4.4*	126 ± 5.3	136 ± 5	129 ± 6
	Hexosamine (mg/100 ml)	131 ± 7.2	120 ± 3.5	134 ± 6.8	136 ± 4.4
Fasted	Body wt (g)	114 ± 4.3	106 ± 5.9	102 ± 2.2	91 ± 6.4
	Hexosamine (mg/100 ml)	126 ± 7	132 ± 6.2	113 ± 4.9	108 ± 2.5

* Stand. error of the mean.

corrected for these hematocrit changes as follows: (Corrected Hexosamine mg/100 ml) =

$$\frac{(100-H_2)\,(\text{observed hexosamine mg/100 ml})}{(100-H_1)}$$

where H_1 = control hematocrit and H_2 = experimental hematocrit. *Plasma volumes* were measured with the dye T-1824(13) following the withdrawal of blood for a hexosamine determination. The final blood volumes were corrected for the prior removal of this sample.

Results. Age. Plasma hexosamine determinations were performed on 4 different age groups of 3 rats each, which had been raised on Purina Chow and kale. The results in Table I show that the hexosamine concentration increased from a low value in the 40 g group (4 weeks) to a high in the 160 g group (11 weeks), and then fell slightly in the 270 g group (16 weeks). These observations demonstrated the need for adequate control rats when studying plasma hexosamine levels in the rapidly growing period.

Fasting. Ten rats were used to determine the effect of fasting on the total plasma hexosamine (mg) (Table II). Five rats were permitted to eat *ad libitum* and food was withheld from the other group of 5. After 2 days, determinations were made of their hematocrits, blood volumes and plasma hexosamine levels. The total circulating hexosamine (mg) was calculated on the basis of observed values for plasma volume (P V) and plasma hexosamine concentration in mg/100 ml (Hex), *i.e.*, $\frac{(P\,V)\,(Hex)}{100}$. There were no significant differences in hexosamine expressed as concentration (mg/100 ml), however, the hematocrit values in the fasted rats increased significantly. The hematocrit changes are shown to be primarily due to a decreased plasma volume (76.0% of total blood volume decrease) and to a lesser extent to a decrease in the red cell volume (24.0% of total blood volume decrease). The total circulating hexosamine (mg), as calculated on the basis of

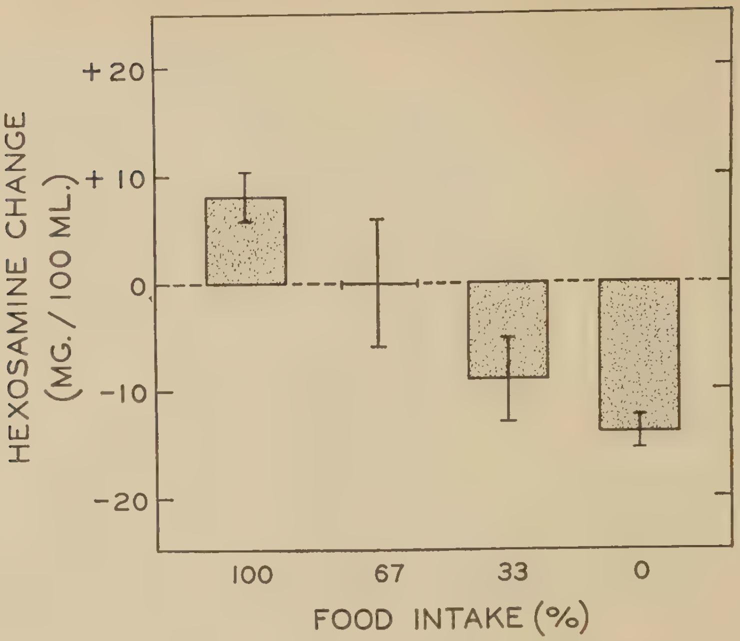

FIG. 2. The effect of different amounts of food intake over a 3 day period on plasma hexosamine levels in the rat. Vertical lines refer to S.E. of the mean.

observed plasma volume and the concentration of plasma hexosamine, were significantly reduced. Frequent plasma samples were obtained over a 2-day period to determine how soon the effects of fasting on hexosamine became manifest and to see if there were any diurnal changes in plasma hexosamine levels. A control group eating *ad libitum* (Purina Chow), and a group fasted from the start of the experiment were used. There were 16 rats in each group, with 4 being used at each time interval indicated in Table III. Each rat was bled only one time. There were no significant diurnal changes in the control group. Fasting did not significantly lower the hexosamine level until 24 hours after the start of the experiment. In another experiment in which rats had access to food and in which plasma was obtained at 9 A.M., 1, 5, and 9 P.M. of the same day, no changes were noted. The time of sampling during the day does not appear to be an important factor.

Food intake. Since fasting lowered the total plasma hexosamine, it became important to know what the critical food intake level was for this effect. Accordingly, 4 groups with 5 rats in each group (95-118 g) were permitted to eat *ad libitum* for a 3-day control period (Fig. 2). The daily average intake (g) for each rat was calculated. The first group continued to eat *ad libitum* (10.0 ± 0.3 g/day); each rat of the second group was fed daily with 66.7% of its calculated daily average of the control period (6.6 ± 0.2 g/day); each rat of the third group was fed daily with 33.3% of its calculated daily control average (3.3 ± 0.1 g/day); the fourth group was fasted. Plasma samples were obtained at the end of the control period and at the termination of the experiment which ran 3 days. The control group (100% intake) developed a slight increase in plasma hexosamine, probably due to the stress of bleeding. The group getting a 66.7% intake had lower hexosamine values some of which were well below the control range. The fasted group and those rats getting 33.3% intake had significantly reduced values. This experiment demonstrated the critical effect of only slight reductions of food intake, and stressed the need for careful dietary controls in plasma hexosamine studies.

Stress. The general plasma hexosamine response to stress is demonstrated in Fig. 3.

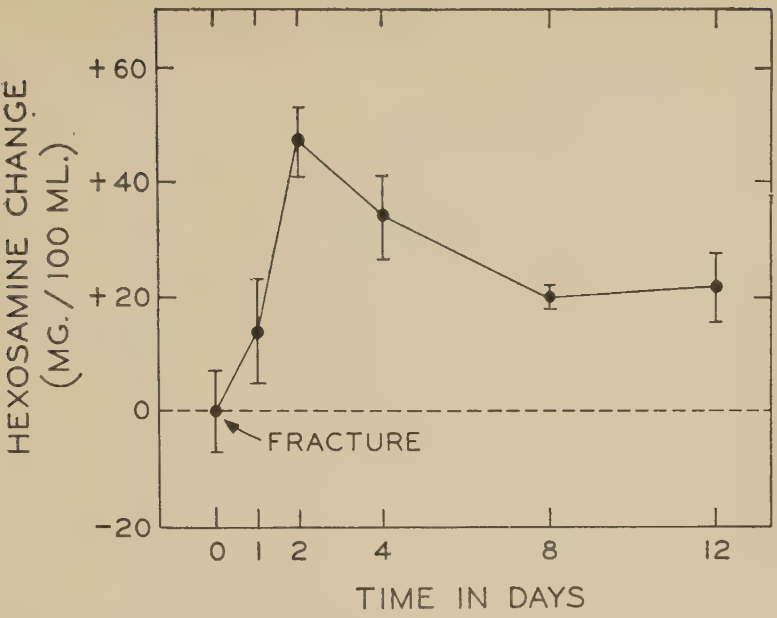

FIG. 3. Effect of fracture on plasma hexosamine levels in the rat. Vertical lines refer to S.E. of the mean.

Six groups of 4 rats weighing 100-130 g were fed Purina Chow *ad libitum*. The right femur and tibia were fractured in each rat under light ether anesthesia. Single bleedings were performed at the indicated time intervals. The plasma hexosamine level increased temporarily reaching a peak at 2 days. Repeated experiments with this and other types of stress (turpentine injections, sham operations, epinephrine, blood-letting) have all shown hexosamine increases, with maximum responses between 2 and 4 days.

Fasting and stress. To study the effect of fasting on stress-induced plasma hexosamine changes, 4 groups of rats, with 5 rats in each group were used. Two groups received 0.4 ml subcutaneous injections of Venice Turpentine in their backs. Of these, one group was permitted to eat *ad libitum* and the other group was fasted. Two control groups were used, one was pair-fed with turpentine-injected rats eating *ad libitum* and the other group was fasted (Fig. 4). The animals were bled serially, and the hexosamine values corrected for hematocrit changes and expressed as mg/100 ml change. The turpentine-injected rats which had access to food, despite a reduced intake following the stress, developed a significant rise in plasma hexosamine. Their pair-fed controls, however, developed an initial fall in hexosamine, probably due to the impaired intake. The rise in hexosamine was not only prevented by fasting the other turpentine-injected group, but was lowered to a level similar to that of the fasted controls.

Discussion. The bound plasma polysaccharides in man consist essentially of hexosamine, mannose and galactose, occurring in approximately equimolar proportions, with a combined plasma concentration greater than 3 times the normal concentration of blood glucose. Measures of these polysaccharides have been obtained in several ways—as "serum polysaccharide"(14), non-glucosamine polysaccharides(15), total mucoprotein(16), hexosamine(1), and others. Regardless of the methods used, it has been repeatedly demonstrated that following many types of stress, clinical or experimental, these substances increase in the plasma(1,17-19). Little is understood concerning the mechanism of these changes. ACTH or cortisone will lower elevated serum hexosamine(6) and serum mucoprotein(20) levels. A thyrotropic hormone fraction produced striking increases in the serum of 2 patients(21). The specificity of

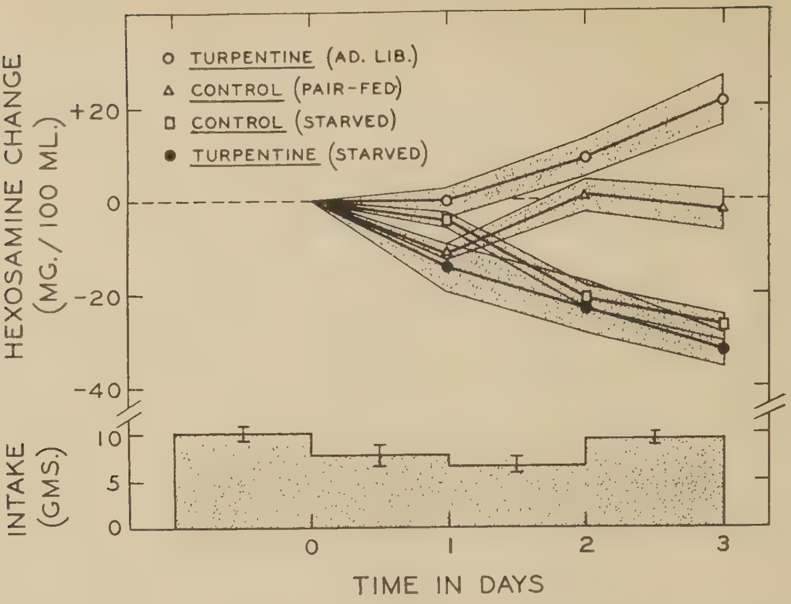

FIG. 4. Effect of fasting on stress-induced (turpentine injections) plasma hexosamine changes. Shaded areas on curves and vertical lines refer to S.E. of the mean.

this response has not been established. It has been suggested, however, that the serum hexosamine response is a stress-induced change mediated by a pituitary hormone, probably related to the thyrotropic hormone fraction(21).

Summary. The following observations were made on plasma hexosamine levels in the rat: 1) there is an increase during the period of rapid growth, 2) no significant diurnal changes could be demonstrated, 3) the amount of food eaten is important in the maintenance of a normal level, 4) stress increases and fasting lowers the plasma hexosamine level in the normal rat, 5) fasting prevents the expected increase following stress.

The authors are grateful to Mr. Joseph B. Foley for his invaluable technical assistance.

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Oral Folic Acid Tolerance Test in Normal Human Subjects and Patients with Pernicious Anemia.* (20011)

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In pernicious anemia, vit. B_{12} appears to produce a complete hematologic and neurologic remission(1). Pernicious anemia seems to represent a conditioned deficiency of vit. B_{12}. Folic acid will also produce a hematologic response in most cases, but patients may experience hematologic or neurologic relapse during treatment with folic acid alone(2). In attempting to explain the action of folic acid in pernicious anemia, one must consider the possibility that a deficiency of this vitamin exists in some cases. Such a deficiency could arise from an insufficient intake, insufficient synthesis by the intestinal flora, absorption or destruction by the intestinal flora, impaired splitting of dietary folic acid conjugates, impaired intestinal absorption, inadequate utilization or rapid destruction in the body, and increased excretion.

These experiments are an attempt to measure the intestinal absorption of folic acid. The balance technic is not applicable to this problem because of the variants introduced by the intestinal flora; accordingly the approach adopted has been to determine the effect of oral dosing upon blood levels; *i.e.*, that of tolerance curves.

Material and methods. Seventeen healthy white adult scientists were used as "normal" subjects. Fourteen were men and 4 women. They ranged in age from 19 to 53 years. Ten patients with well-established pernicious anemia were studied. Five were male and 5 female, and one of the males was a Negro. They ranged in age from 46 to 78 years. Six of the patients had been maintained in remission by intramuscular liver extract[‡] for several years and one by vit. B_{12}[§] for several years. Three patients were in the early stages of a hematologic response to B_{12} and were anemic when the test was performed. *Folic acid*[‖] was given one to 2 hours after breakfast as a freshly prepared aqueous suspension. Venous blood was collected prior to and at intervals after ingestion. The serum was separated and frozen within one to 2 hours after venipuncture. For assay, the serum was diluted with water and assayed microbiologically(3) with *Streptococcus fecalis* ATCC 8043. The assays were read titrimetrically after incubating 72 hours and steaming 10 minutes, because it was found that autoclaving and the quaternary amine used for a germicide caused turbidity of the serum. Recoveries using 3 dilutions of one or 2 serum samples were included with each assay, and no assay was accepted unless these recoveries were between 90 and 115% and the standard curve approached a straight line when plotted on log-log paper. Twenty-four hour urine specimens were collected under toluene from certain of the normal subjects beginning at the time of folic acid ingestion. An aliquot was frozen and later assayed in a fashion similar to that used for serum, except that incubation was continued for only 18 to 24 hours and the assays read turbidimetrically.

Results. The serum level of folic acid prior to folic acid ingestion was 4 mγ (millimicrograms) per cc or less in every subject but one, who 48 hours after omitting a daily dose of 5 mg of folic acid, had a level of 6 mγ per cc. These levels were found to be uniformly low whether or not the subject had eaten breakfast.

Table I shows the highest serum levels

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[*] Supported in part by funds made available by the National Vitamin Foundation, the Nutrition Foundation, and The Rockefeller Foundation.

[†] Fellow in the Medical Sciences, National Research Council. Present address: Naval Medical Research Institute, Bethesda, Md.

[‡] Liver extract was generously supplied by The Wilson Laboratories, Division of Wilson and Co., Inc.

[§] Crystalline vit. B_{12} was made available through the courtesy of Merck and Co., Inc.

[‖] Folic acid was supplied through the generosity of the Lederle Laboratories Division of the American Cyanamid Co.

TABLE I. Maximum Values of Serum Folic Acid Reached Following Folic Acid Ingestion.

	.5 mg dose			1 mg dose			2 mg dose		
Subject (healthy)	Max, mγ/cc	Time of peak hr after ingestion	Activity 24-hr urinary excr., γ	Max, mγ/cc	Time of peak hr after ingestion	Activity 24-hr urinary excr., γ	Max, mγ/cc	Time of peak hr after ingestion	Activity 24-hr urinary excr., γ
C, Sr.				0	—	—			
W	1	1	6	2	4	—	40	1	140
McL	2	1		2	4	56	27	4	
C	—	—	21	4	1	36	40	2	465
D				4	1	46			
DC				7	2	—			
CD				7	4	26	10	4	114
B				10	6*	34	46	1	235
O	2	1	8	18	4	36	31	2	240
E	1	1	7	20	4	23			
F				36	6*	46			
N	1	4	11						
P	5	4	5				28	4	220
Mc							18	6*	200
DB							19	4	180
U							25	6*	165
GB							33	4	165
Subject (pernicious anemia)									
K				2	2*	—			
E				4	1	—			
T				4	1	—	4	1	180
C				8	2	—			
B				10	1	—			
G				12	1	—			
M				14	1	—			
V				16	1	—			
P				24	1	—			
F							19	1	455

* Last sample drawn.

measured in each test following the ingestion of 0.5 to 2.0 mg of folic acid. Twelve of the healthy subjects attained a maximum within 4 hours and 4 showed a higher level at 6 hours. An illustrative course of blood serum levels following the ingestion of 1.0 mg by one healthy subject is given in Fig. 1.

One mg appeared to be the critical dose at which some of the normal subjects showed an increase in serum level sufficient to be regarded as evidence of absorption. It was, therefore, chosen as a standard dose for the patients with pernicious anemia. Six of the healthy subjects whose serum level did not rise above 10 mγ per cc were given 5 mg folic acid daily for one week and the 1 mg tolerance test repeated 48 hours after the last dose. Table II demonstrates that each subject showed a somewhat higher peak in the second test, but the increase was not striking.

The 24-hour urinary excretion of folic acid by several of the normal subjects following varying doses of folic acid are also presented in Table I. Difficulties were encountered in assaying these urines. Recoveries from individual samples varied from 50 to 200%, using both titrimetric and turbidimetric assays. Therefore, the urinary excretion is expressed as "folic acid activity" in recognition of this limitation.

Nine of the patients with pernicious anemia were given 1 mg of folic acid and blood samples collected before, and 1, 2, and 3 hours after ingestion. Two of the patients were given 2 mg and blood samples collected before, and 2, 4, and 6 hours after ingestion. The peak values reached did not differ significantly from those of the normal subjects, but the peaks appear to occur earlier than in the normal subjects (Table I).

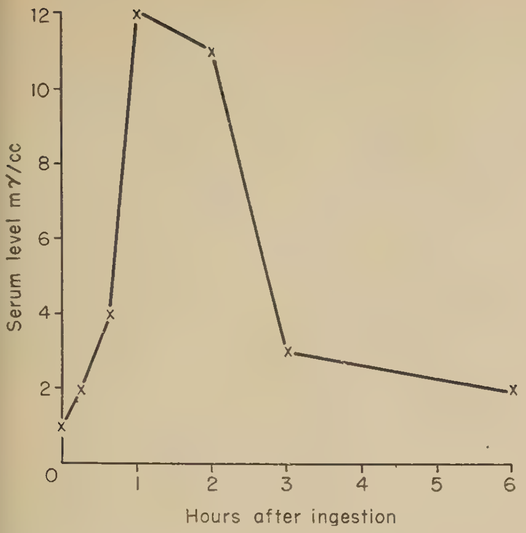

FIG. 1. Blood Serum levels of folic acid following ingestion of 1 mg of folic acid. The subject had received 5 mg of folic acid daily for 7 days ending 48 hr prior to this test. Male, 31 years old.

Discussion. The reproducible results and good recoveries obtained with the serum assays indicate a degree of accuracy that permits measurement of the slight increases in serum levels encountered in this study. It seems reasonable to assume that the increases measured represent free folic acid in the serum because they occurred following folic acid ingestion, because *S. fecalis* is unresponsive to folic acid conjugates(4), and because the serum was frozen shortly after collection, to prevent the release of bound folic acid by serum enzymes.

This study confirms previous reports(5) that the normal diet does not cause a significant rise in serum levels of free folic acid and justifies the use in such studies of the postprandial state. Furthermore, these data fail to indicate that a meal previous to ingestion causes delay in absorption.

The appearance of free folic acid in the serum as early as one hour after ingestion indicates that this vitamin is rapidly absorbed. The failure of some of the normal subjects to show an increased serum level following ingestion of 1 mg of folic acid may signify delayed absorption, more rapid conversion to "bound" folic acid in the blood, rapid uptake by the tissues due to tissue desaturation, or an increased rate of excretion. The 24-hour excretions did not differ between those subjects whose serum level rose and those showing no increase. The small increase in the maximum level attained when the tolerance test was repeated following a period of 5 mg of folic acid per day suggests that unsaturation of the tissue is not the factor determining the height of the tolerance curves in these subjects. Therefore, the observed differences in maximum levels of folic acid in the serum following a standardized dose seem to reflect primarily variations in the rate of absorption.

The urinary excretions do not vary importantly from those found in other studies (6), and fail to account for most of the ingested dose. Activators and inhibitors present in some urines cause inaccuracies in the assay.

The data of this study indicate that there is no abnormality in the intestinal absorption of folic acid by patients with pernicious anemia. The fact that oral and parenteral folic acid are equally effective in treating pernicious anemia(7) supports this conclusion, as does the finding of Castle(8) that intrinsic factor did not enhance the hematopoietic effectiveness of oral folic acid in pernicious anemia.

The fact that peak levels were reached later and high levels maintained longer in the normal subjects than in the pernicious anemia patients suggests that the latter group removed free folic acid from the blood more rapidly. This could be due to a relative deficiency of folic acid and tissue desaturation (or more avid binding of folic acid in the

TABLE II. Change in the 1 mg Tolerance Test Produced by the Ingestion of 5 mg of Folic Acid Daily for 1 Week.

	Peak level of serum folic acid reached after ingestion of 1 mg of folic acid	
Subject	Original test, mγ/cc	Peak after 5 mg folic acid daily for 1 wk, mγ/cc
C	4	9
W	2.5	12
B	10	13
D	4	6
McC	2	5
CD	7	8

blood). Such a theory is reinforced by the observation that 2 of the normal subjects given 5 mg of folic acid daily for a week showed more prolonged high levels and a later peak in the 1 mg tolerance test after this "saturating dose" than before.

From these limited observations, the oral folic acid tolerance test appears to be a useful tool in the study of folic acid metabolism. Additional studies of the level and duration of serum folic acid increases should provide evidence concerning the rate of folic acid absorption as well as the rate of its removal from the blood in suspected deficiency states.

Summary. An oral folic acid tolerance test is described and its limitations discussed. Evidence is presented that the test is useful in assessing the absorption of folic acid. No defect in the gastrointestinal absorption of folic acid in patients with pernicious anemia has been demonstrated.

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Thyroid Function in Hypophysectomized Mice Bearing Intraocular Pituitary Implants. (20012)

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(Introduced by Roy Hertz.)

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There are conflicting reports(1-8) on the degree of secretory activity of the mammalian pituitary transplanted from its normal site. Most investigations were conducted with animals of unspecified genetic constitution. Since genetic heterogeneity may have affected the viability of the transplants, it was felt that a reinvestigation of the problem utilizing a genetically homogeneous group of animals might be of value. In the present experiments, intraocular pituitary transplants maintained a level of thyroidal radioiodine metabolism much higher than that of hypophysectomized controls but had no significant effect on body weight or on the weight of the thyroid, adrenals, ovaries, or uterus.

Materials and methods. BALB/c x C_3H mice were used in all experiments. As F_1 derivatives of 2 closely inbred lines, transplantation among them produces no incompatibility reaction and implants persist indefinitely. Two-month-old females were used as hosts except in Exp. VII, where 4-week-old females were implanted. In one experiment, not included in Table I, males were used as hosts. There was no indication of a sex difference in the response. Donors were embryos at the 14th or 15th day of gestation (Exp. I, II, VI, VII) or 1-2 day old newborn mice (Exp. III, IV, V) irrespective of sex. A single pituitary was transplanted into each animal except in Exp. IV, in which 4 pituitaries were implanted, 2 into each eye. The pituitaries were placed into the anterior chamber by a method previously described(9). Exp. VIII was designed to test the potency of adult pituitaries. Here 3-month-old females were donors and 1/3 pituitary was placed in each

TABLE I. Effect of Pituitary Implants on Thyroid Function and Target Organ Weight.

Exp. # and group[*]		No. of animals	Age at autopsy, wk	Terminal body wt	Ovaries wt, mg	Uterus wt, mg	Adrenal wt, mg	Thyroid wt, mg	T/S ratio of I^{131}	Total thyroidal I^{131} uptake, %[‡]
I	C	4	14	22.5 ± .8[†]	12 ± 1.3	119.5 ± 19.1	6 ± .4	2.4 ± .1		100 ± 9.3
	H	5		18.2 ± .5	3.2 ± .2	22.2 ± 1.2	2.6 ± .2	1.5 ± .1		.9 ± .2
	I	6		18.6 ± .9	3.4 ± .5	22.7 ± 1.8	2.8 ± .2	1.6 ± .1		33.6 ± 2.5
II	C	6	13					2.1 ± .1		100 ± 13.3
	I	5						1.4 ± .1		27.3 ± 2.8
III	C	13	14	23 ± .3				2.4 ± .1		100 ± 6.3
	H	11		18.6 ± .6				1.6 ± .1		2.1 ± .2
	I	13		20.4 ± .5				1.7 ± .1		47 ± 4.1
IV	C	4	18	25.5 ± .6	15 ± .6	154.1 ± 18	6.5 ± .1	2.6 ± .1		100 ± 15.4
	H	5		16.7 ± 1.1	1.8 ± .1	15.1 ± 2.2	2.1 ± .2	1.1 ± .1		1.9 ± .9
	I	2		19 ± 1	1.8 ± .3	17.1 ± 1.8	2.3 ± .1	1.5 ± .2		41.1 ± 3.2
V	C	6	15	22.7 ± .3		120.4 ± 15.5		2.5 ± .1	265 ± 20.2	
	H	6		16.2 ± .3		18.6 ± .3		1.4 ± .1	29 ± 4.6	
	I	11		17.3 ± .3		18.6 ± .6		1.5 ± .1	175 ± 18.1	
VI	C	6	13	23.2 ± 1			5.6 ± .2			
	H	6		18.5 ± .6			2.7 ± .2			
	I	4		19.1 ± .6			2.5 ± .2			
VII	C	4	9	19.1 ± .8	7.2 ± .8	56.7 ± 10	4.7 ± .1	1.8 ± .1		
	H	3		12.9 ± .3	1.6 ± .2	8.8 ± 1.7	1.7 ± .1	.9 ± .1		
	I	4		14.6 ± .6	1.6 ± .1	9.1 ± .9	2.1 ± .1	1.2 ± .2		
VIII	C	6	15	23.8 ± .5		156 ± 15.2		2.5 ± .1		100 ± 5.4
	H	11		21.5 ± .3		21.3 ± .3		1.9 ± .1		2.5 ± .2
	I	8		19.4 ± .3		21.8 ± .4		2 ± .1		32.2 ± 2.9

[*] C = intact controls, H = hypophysectomized controls, I = hypophysectomized-implanted mice.

[†] Stand. error.

[‡] Thyroidal I^{131} uptake is expressed as total uptake for entire thyroid gland. For comparison the uptake of intact controls in each exp. is given as 100% and other figures are relative to this standard. The absolute thyroidal accumulation of intact controls averaged 10-15% of administered dose at end of 4 hr.

eye of the host. In all experiments the animals were hypophysectomized by the parapharyngeal approach one week after implantation. Control groups consisted of unoperated and hypophysectomized mice of the same age. Three weeks after hypophysectomy the animals were utilized in the definitive experiments.[*] To determine *total accumulation of radioiodine,* each animal was injected with a small tracer dose of I^{131}, ranging from 0.05 to 1 μc in the individual experiments. All animals in a given group were killed by exsanguination at the same interval after radioiodine injection—either 3½ or 4 hours. After weighing, the thyroid was placed on a filter-paper-covered flat copper planchet, the whole enclosed in Scotch tape, and the thyroid smashed flat by drawing a heavy weight over it. Radioactivity of the sample was determined by beta-counting with a mica end-window G-M tube. The *thyroid/serum iodide ratio,* comparing the concentration of I^{131} as iodide in the thyroid with that of the serum, was determined by a method similar to that previously described for the rat(10). Each mouse received a subcutaneous injection of 2.5 mg propylthiouracil 30 minutes before the administration of a tracer dose of radioiodine. The animals were killed by exsanguination 2 hours after the I^{131} injection. Radioactivity per unit weight of thyroid was then compared to that of the serum. The uteri were weighed on a 0-200 mg Roller-Smith torsion balance. All other organs were weighed on a 0-15 mg Roller-Smith balance.

Results. No significant anterior lobe function could be detected from the weight of the various target endocrine organs or from body weight. As seen in Table I, thyroid, adrenals, ovaries, and uterus were equally atrophic in the hypophysectomized control and hypophysectomized-implanted animals. In marked contrast to this was the ability of the thyroid of the implanted animals to accumulate I^{131}. Intraocular pituitary tissue enabled the thyroid to accumulate radioiodine to levels 20-30 times that of hypophysectomized controls and 2/3 that of intact controls per unit thyroid weight. Similarly, the implanted animals had a much higher thyroid/serum iodide ratio than their hypophysectomized controls, but again this was only 2/3 that of the intact controls.

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[*] The animals were fed the following pellet diet: skim milk powder, 22.75; ground whole wheat, 61.52; brewer's yeast (dried), 4.00; cod-liver oil, 2.00; sodium chloride, 1.40; ferric citrate, 0.13; corn oil, 8.20.

No difference resulted when either embryonic, newborn, or adult donors were used, or when two-thirds, one, or 4 pituitaries were implanted.

Discussion. These results indicate that thyroidal concentration and uptake of iodine can vary independently of thyroid weight. Apparently an intraocular pituitary implant in the mouse is able to maintain approximately normal iodine metabolism by the thyroid gland, as measured by the thyroid/serum iodide ratio and by total thyroidal accumulation of I^{131} at the end of 4 hours. It is not, however, able to maintain thyroid weight significantly above the values of hypophysectomized controls.

Experiments in the rat have previously indicated that thyroid weight and follicle cell height vary independently of the thyroid/serum ratio(10). Anterior hypothalamic lesions in this animal prevent any increase in thyroid size due to chronic thiouracil administration but do not prevent the usual 10-fold increase in thyroid/serum iodide concentration(11). This indicates that integrity of the anterior hypothalamus may be necessary for the pituitary to maintain the weight and cell height of the thyroid. The present data indicate further that the pituitary of the mouse may maintain thyroidal iodine metabolism in the absence of direct hypothalamic connections.

Summary. Intraocular hypophysial transplants in hypophysectomized mice did not maintain body weight or the weights of thyroid, ovaries, adrenals, or uterus significantly above those of hypophysectomized controls. Nevertheless, the implants were able to maintain a radioiodine uptake per unit thyroid weight and a thyroid/serum iodide ratio at 2/3 the level of the intact controls. This indicates that thyroid function and thyroid size are not necessarily correlated in the mouse and that the former can be maintained to considerable degree in the absence of the normal hypophysial anatomical relationship.

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Fate of Implanted Collagen in the Liver of White Rats. (20013)

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Observations of several investigators suggest that the quantity of fibrous tissue in the liver of animals with experimental cirrhosis may diminish spontaneously(1,2) or may be influenced by favorable nutritional conditions (3-5). A study of the reversibility of fibrosing processes is hampered by the variability in the development and intensity of experimental cirrhosis in the individual animals or any experimental series.

The present study was undertaken to find out whether collagen can be absorbed in the liver. For this purpose a standardized procedure was employed whereby a) a known quantity of collagen was available for absorption by the liver; b) the process could be accurately dated; and c) extrahepatic factors could be eliminated, such as general symptoms of poisoning, disturbances of food intake and utilization or alterations of the hepatic vasculature.

Material and methods. In 22 male rats between 180-240 g a single collagen thread attached to a non-traumatic needle was implanted transversely into the large left lobe of the liver. Twelve rats received flexor or extensor tendons which were removed from the hind feet of a freshly killed young adult rat and immediately implanted. Ten rats received plain surgical gut, U.S.P. (sheep collagen), size 3/0 which was supplied heat sterilized and sealed in ethyl alcohol containing mercuric iodide of 1:2000. Before use the threads were washed with sterile physiologic saline. Only minimal hemorrhage resulted from the operative procedure and all animals recovered immediately following closure of the abdomen. The animals of both series were sacrificed in pairs at 1, 2, 3, 4, and 8 weeks. In addition, rats with implanted tendon were sacrificed 12 weeks after the operation. Two blocks of tissue from the left lobe of each liver were examined histologically. Sections were stained with hematoxylin and eosin and Laidlaw's silver method counterstained with van Gieson.

Results. 1. *Implantation of homologous tendon.* The tendon remained unchanged in size and apparently viable during the first 2 months of observation. In one animal sacrificed at the end of this period extensive calcification appeared in the tendon (Fig. 1). In one rat examined at the end of 3 months the tendon appeared still viable but slightly narrower in diameter than in all other animals. With regard to the absence of regressive changes or of invading granulation tissue we would consider this difference in size to be due to smaller caliber of the tendon at the time of implantation as compared with the rest of the tendons. A narrow capsule of collagen tissue separated the tendons from the hepatic parenchyma in the livers of all rats. The adjacent parenchyma showed no evidence of compression or cytological alterations.

2. *Implantation of surgical gut.* The implanted surgical gut excited a reaction in the liver characteristic of a foreign body. At first it was surrounded by a wall of neutrophilic leukocytes, cellular granulation tissue and foreign body giant cells. A wide zone of fibroblasts had formed at the periphery between which were numerous concentrically arranged argyrophile fibers, most dense adjacent to the hepatic parenchyma (Fig. 2). Within 2 weeks the gut was fragmented and invaded by granulation tissue and foreign body giant cells. In the periphery a capsule of collagen fibers had developed which showed silver-impregnated fibrils in lesser amount than before.

By the third week the thread of gut was in greater part replaced by a cellular granulation tissue including a small number of collagen fibers and surrounded by scarce concentric

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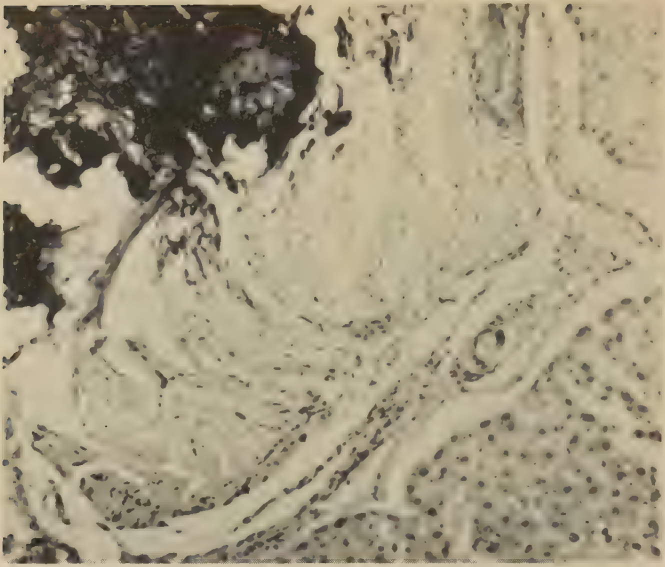

FIG. 1. Homologous tendon in liver, 2 months following implantation. The tendon is in part calcified and surrounded by a narrow rim of newly formed connective tissue. Hematoxylin and eosin. × 150.

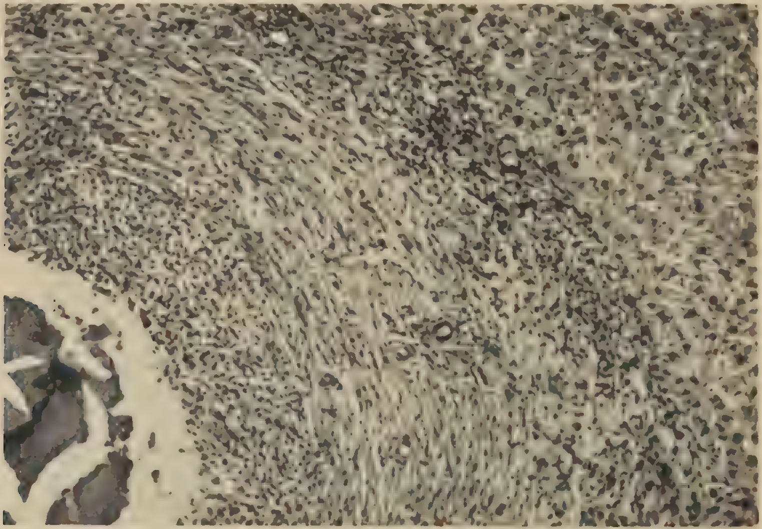

FIG. 2. Surgical gut in liver one week following implantation. A wide capsule has formed around the gut consisting of abundant fibroblasts and argyrophile fibers. Hematoxylin and eosin. × 150.

bundles of argyrophile fibrils (Fig. 3).

During the remainder of the observation period the granulation tissue was gradually converted into a small nodule of loose fibrous tissue containing a few hemosiderin-filled macrophages (Fig. 4).

Discussion. Homologous collagen in the form of tendon is apparently an inert material and excites little or no reaction from the hepatic tissue. It remains unchanged in size and shape after implantation into the liver. This part of the present experiment, therefore, does not indicate whether fibrous tissue formed in the liver may regress spontaneously or not.

Surgical gut (sheep collagen), on the other hand, is rapidly replaced by invading granulation tissue and foreign body giant cells. The significant feature of this process is that

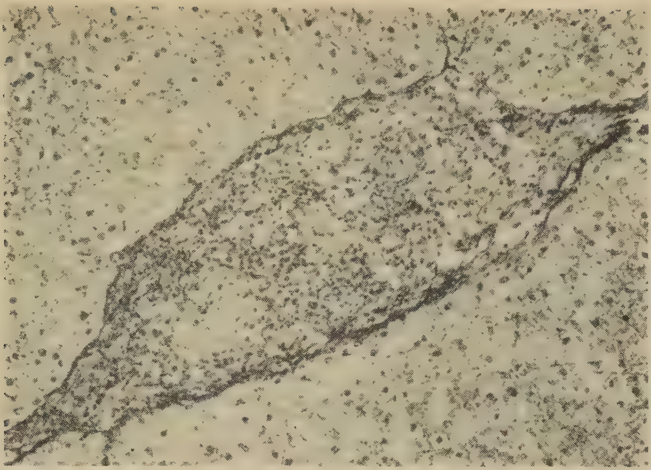

FIG. 3. Three weeks following implantation the gut is almost entirely absorbed. Only a few reticulum fibers are still present. Laidlaw-van Gieson's stain. × 150.

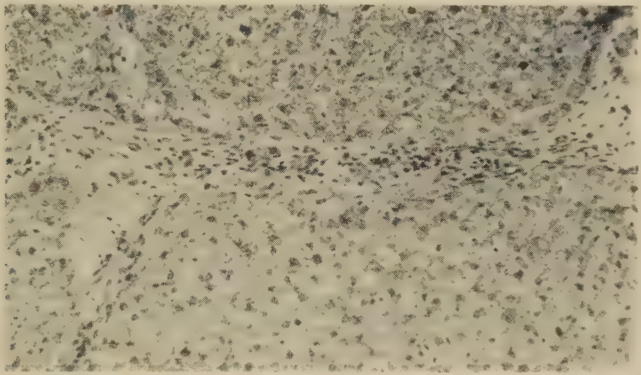

FIG. 4. Two months following the operation a small, loose scar had remained at the site where surgical gut was implanted. Hematoxylin and eosin. × 150.

initially, up to one week following the operation, there is proliferation of fibrous tissue around the foreign "gut" which later almost entirely disappears. The wide zone of reticulum and collagen fibers which surround the gut are probably due to actual proliferation and not to a condensation of preformed connective tissue.

The regression of the newly formed fibrous tissue demonstrated in the present experiments supports the observations on the possibility of spontaneous "reversal" of experimental cirrhosis in the liver of rats which has been demonstrated by quantitative chemical determinations by Morrione(6).

Summary. 1. Homologous tendon and plain surgical gut were implanted into the livers of young adult rats for the purpose of investigating the capacity of the organ of absorption and new formation of collagen fibers at measured intervals. 2. Homologous tendon caused no remarkable reaction in the liver and remained viable and in undiminished quantity for an observation period of 3 months. 3. Surgical gut was absorbed during about 3 weeks. At the end of the first weeks there appeared abundant newly formed collagen fibers around the implanted gut. These were observed in decreasing quantity during the following 7 weeks. 4. It appears that the normal liver has the capacity to absorb newly formed collagen fibers as well as non-viable collagen (surgical gut), but not homologous tendon collagen.

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Competitive Inhibitive Action of Normal Fatty Acids on Mammalian D-Amino Acid Oxidase. (20014)

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During an investigation of the effect of various carboxylic compounds on the metabolism of mammalian tissues it was found that some of the simple normal fatty acids are competitive inhibitors of the mammalian D-amino acid oxidase. This action of the simple fatty acids does not seem to have been reported although Klein and Kamin(1) noticed an inhibition of D-amino acid oxidase by sodium benzoate. Later Bartlett(2) showed that the inhibition was competitive and that of many derivatives tested *m*-methyl benzoate was most effective. Zeller and Maritz(3) found that benzoate also inhibits ophio-L-amino acid oxidase and that other aromatic carboxylic and sulphonic acids had similar effects. The short chain normal fatty acids are shown to be more specific in their action; they inhibit the mammalian D-amino acid oxidase but have no action on ophio-L-amino acid oxidase.

Materials and methods. The source of the mammalian enzyme was a rat kidney homogenate. The animal was decapitated, exsanguinated, the kidneys removed and chilled on cracked ice. They were defatted, cut into small pieces and homogenized in 7.0 ml ice cold saline for 2 minutes. The homogenate was filtered through two layers of cheesecloth and 1.0 ml used per vessel. The substrate in all experiments reported was DL-methionine. The results were confirmed in most cases with DL-alanine as substrate, and it was shown that L-alanine was not oxidised by the rat kidney homogenate. The activity of the amino acid oxidase was followed in the Warburg apparatus by measuring oxygen uptake at 37°C with KOH filter paper in the centre well. In general, constant rates of oxygen uptake were found for 20 minutes and the inhibitions reported are inhibitions of this initial constant rate of oxygen uptake. Dried cobra venom was obtained from Hynson, Westcott and Dunning Ltd. (Baltimore) and 3.0 mg was used per vessel as the source of the ophio-L-amino acid oxidase.

Results. All the normal fatty acids from acetic to octanoic were tested as inhibitors of

TABLE I. Oxidation of DL-methionine by Rat Kidney Homogenate in Presence of the Sodium Salts of the Short Chain Fatty Acids.

Additions*	μl oxygen taken up in 20 min.	% inhibition
Nil	23	0
Methionine	146	
Sodium acetate	18	0
" " + methionine	145	
" propionate	18	0
" " + methionine	141	
" butyrate	24	39
" " + methionine	100	
" pentanoate	18	69
" " + methionine	56	
" hexanoate	15	50
" " + methionine	76	
" heptanoate	16	57
" " + methionine	69	
" octanoate	14	7
" " + methionine	128	

* All vessels contained 1 ml rat kidney homogenate, 1 ml .10 *M* phosphate buffer pH 7.4 and water to make a final vol of 3 ml. All the sodium salts were present at a final concentration of .01 *M* and the methionine at .02 *M*.

rat kidney D-amino acid oxidase; the results are shown in Table I. Sodium pentanoate was the most effective inhibitor and both the highest and lowest chain length fatty acids tested were ineffective. The nature of the inhibition by the sodium pentanoate was investigated using varying quantities of substrate and inhibitor. The results, plotted by the method of Lineweaver and Burk(4), are shown in Fig. 1. The inhibition is obviously competitive, the K_m for D-methionine in this system being approximately 8 x 10^{-3} M and the molar ratio of D-methionine to sodium pentanoate for 50% inhibition is of the order of 7.5:1.

In contrast to its effect on D-amino acid oxidase, sodium pentanoate failed to inhibit the ophio-L-amino acid oxidase of dried cobra venom even when the molar ratio of DL-methionine to pentanoate was 1:1. (Table II). The observed effects are therefore more specific than those of benzoate which inhibits both the kidney and the venom enzymes.

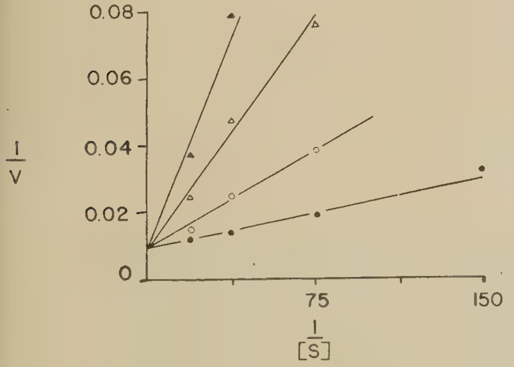

FIG. 1. Competitive inhibition of DL-methionine oxidation by sodium pentanoate. The velocity (V) is expressed as μl oxygen taken up in 12 min. (corrected for the endogenous respiration) and the DL-methionine concentration [S] is expressed as molarity. The reciprocals of these values are plotted. ● No sodium pentanoate present, ○ 3.3×10^{-3} M, △ 1×10^{-2} M, ▲ 2×10^{-2} M sodium pentanoate present. Other conditions as in Table I.

TABLE II. Oxidation of DL-methionine by Cobra Venom in Presence of Sodium Pentanoate.

Additions*	μl oxygen taken up in 60 min.
Nil	11
Methionine	167
Sodium pentanoate	12
" " + methionine	161

* All vessels contained 3 mg crystalline cobra venom, 1 ml .10 M phosphate buffer pH 7.4 and water to make a final vol of 3 ml. The final concentration of both methionine and sodium pentanoate was .02 M.

Summary. Some of the short chain normal fatty acids are shown to be competitive inhibitors of the D-amino acid oxidase of rat kidney. In contrast to benzoate, which is a competitive inhibitor of this enzyme and of the ophio-L-amino acid oxidase of cobra venom, sodium pentanoate inhibits only the kidney enzyme.

Grateful acknowledgement is made to the National Cancer Institute of Canada for a grant in aid of a programme of work of which the above represents a part, and to Dr. J. H. Quastel of this Institute for his continued interest in this work.

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Differences between Oral and Genital Strains of Human Pleuropneumonia-like Organisms.*† (20015)

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Pleuropneumonia-like organisms have been isolated from various locations of the human body: from the genitourinary tract(1), buccal cavity(2), intestinal tract(3), blood, spinal fluid(4) and from suppurative lesions(1). In our experience they grow abundantly from the debris scraped from between the teeth and from material expressed from the crypts of the tonsils. These organisms present the characteristic morphology of the pleuropneumonia group, but they vary considerably in their properties. Three types isolated from mice differed in serological and pathological properties(5). Recently Edward distinguished 2 different types among strains isolated from the vagina of cows(6) and 3 different types among the strains isolated from dogs(7). The observations presented here suggest that at least 2 different types should be distinguished in human strains.

Cultures of pleuropneumonia-like organisms obtained from specimens taken from the genitourinary tract grow in two forms. That most often observed grows to fairly large colonies (0.1-0.5 mm) and grows abundantly on ascitic agar plates. The appearance of the colonies is variable on the original plates as a consequence of different development and vacuolization of the large bodies on the surface. These differences disappear in subcultures. Cultures obtained from certain *specimens* are *markedly different*. In these the colonies are very small (0.01-0.05 mm) both in the original plates and in transplants and the growth is very poor. Whether these colonies represent a variant of the usual strains or a different species cannot be decided until a better method for their cultivation is devised. The cul-

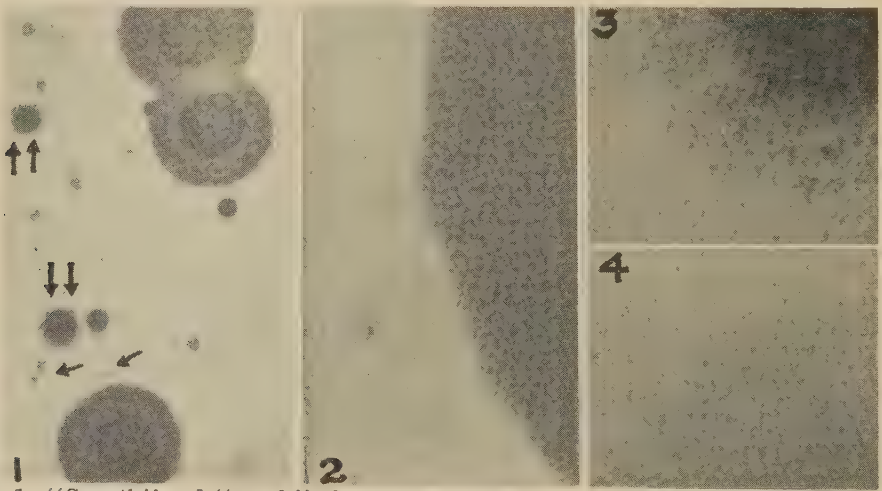

FIG. 1. "Smooth" and "rough" pleuropneumonia-like colonies on a horse-serum agar plate containing penicillin, inoculated from tooth scrapings. The culture was stained with methylene blue. 1. The large "smooth" and small "rough" colonies with low magnification (× 100). Tiny "rough" colonies are marked with a single arrow and fully developed "rough" colonies are marked with two arrows. 2. Edge of a "smooth" colony. Oil immersion. (× 900.) No structure is visible in the colony. 3, 4. Edges of two "rough" colonies. Oil immersion. (× 900.)

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tures obtained from the *buccal cavity* also grow in 2 morphological types both of which are different from the genital strains. Under appropriate conditions the colonies usually isolated grow to a larger size than the colonies of genital strains and differ from the latter by their peculiar "smooth" appearance. The surface growth appears with the low power of the microscope as a homogeneous mass. The uniform small granules which produce the colonies can be observed easily in impression preparations. Large bodies are produced on the surface but they are not vacuolized and are filled with uniform granules.

These relatively *"smooth" colonies* can be seen fairly easily between the bacterial colonies if the plates are inoculated with high dilutions of tooth scrapings. They grow abundantly if the plates are heavily inoculated and bacterial growth is suppressed by penicillin. In addition, sometimes in large numbers, much smaller pleuropneumonia-like colonies develop. These tiny colonies differ in appearance both from the other oral strains and from genital strains. They are surrounded after 2-3 days with a peripheral surface growth and although they are very small they present the characteristic appearance of colonies of the pleuropneumonia group. The peripheral growth consists of large bodies but they are soon vacuolized giving a rough appearance to the colonies. It is difficult to induce growth of these colonies in transplant. After repeated transfers their growth becomes more abundant but the colonies retain their "rough" appearance.

The characteristic *morphological differences* between the genital and oral strains remain unchanged during cultivation. There is also some difference in their nutritional requirements. The "smooth" oral strains grow well on the usual horse blood agar plates while most genital strains grow poorly or not at all. There is also a marked difference in the serological properties of the strains. We found the easily cultivatable genital strains to be serologically similar, as reported by Norman, Saslow and Kuhn (8). There are some irregularities in the reactions of the strains with different antisera but it is uncertain whether these really indicate serological differences. On the other hand,

TABLE I. Agglutination Reactions of Genital and Oral Strains of Human Pleuropneumonia-Like Organisms.

	Antigens																			
	Genital strain "50"					Genital strain "Campo"					Oral strain "smooth"					Oral strain "rough"				
Rabbit antisera	1:20	40	80	160	320	1:20	40	80	160	320	1:20	40	80	160	320	1:20	40	80	160	320
Genital strain "50"	—	3+	3+	3+	3+	—	+	2+	2+	+	0	0	0	0	0	—	0	0	0	0
Oral strain "smooth"	0	0	0	0	0	—	0	0	0	0	3+	3+	3+	2+	2+	—	2+	2+	2+	2+

0 indicates negative reaction; — indicates not done.

The rabbits were treated with cultures grown in broth or on agar plates containing rabbit serum. For the agglutination test, the sediment of broth cultures or washings from agar cultures were used. The test was performed in .4 cc volume in tubes measuring 10 × 75 mm. The tubes were incubated overnight in a water bath at 37°C.

three oral strains, two "smooth" and one "rough", gave no reaction with agglutinating sera produced by 4 different genital strains and the latter were not agglutinated by the sera produced with the oral strains. Two of these strains, "Campo" and "50" were studied by Norman, Saslow and Kuhn. A fifth genital strain was an exception, giving a slight cross reaction with the oral strains. An example of the reactions observed is presented in Table I.

Serological examination of the *"rough" oral strains* remained inconclusive because in several trials we were unable to produce agglutinating sera with them. However, emulsions of a "rough" strain gave strong agglutination with the sera of the "smooth" strain. This would suggest that the "rough" colonies are probably a variant of the "smooth" ones, although transformation of one strain into the other has not been observed.

In addition to the pleuropneumonia-like colonies, L colonies similar in appearance to those developing from various bacteria develop occasionally on the plates if penicillin is added to the media. These colonies usually grow to a larger size and their morphology also distinguishes them from pleuropneumonia-like colonies. In one case we isolated a small Gram negative bacillus from L colonies developing on the plates. The bacillus produced similar L colonies in the presence of penicillin. In another case a Clostridium was present in the specimen and produced L colonies. It is necessary to bear in mind that colonies of various types, some of which may be quite similar to pleuropneumonia-like colonies, may develop on the plates.

One strain of pleuropneumonia-like organisms isolated from spinal fluid(4) and several strains isolated from pathological processes associated with the buccal cavity corresponded in the appearance of the colonies to the "smooth" oral types. The strains isolated from suppurative lesions of the female genital tract corresponded to the genital type.

Conclusion and summary. Pleuropneumonia-like strains isolated from the human genitourinary tract and from the buccal cavity, respectively, differ in the appearance of the colonies, in serological properties and, to a certain extent, in nutritional requirements. It is likely that they represent different species. In addition to the easily cultivatable strains, colonies of much smaller size develop occasionally on the plates inoculated from either source. These small colonies retain their identity in transfer. It is uncertain whether they are variants of the usually cultivated strains.

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Survival Study of Thermally Injured Rats Treated with Piromen®.* (20016)

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In a previous study it was demonstrated that the Arthus phenomenon in rabbits was initially enhanced by administration of the bacterial polysaccharide complex, known as Piromen®, although it was suppressed by administration of cortisone. It was noted at that time that the skin lesions in the Piromen-treated rabbits healed more readily and the scars presented an appearance different from those in the control animals(1). This observation, coupled with other information obtained from studies of the healing of transected spinal cords and inhibition of glial-barrier formation in animals(2,3), lead to the study of the effect of this drug on thermal injuries.

Our initial experiments consisted of spot burns in cats and guinea pigs. The results of this study have been reported in preliminary form(4). Histological aspects of the healing of sublethal injuries of this type are still under investigation. During these studies, the question arose as to whether a bacterial polysaccharide complex might affect beneficially the rate of survival of animals receiving large body-surface lesions. Therefore, it was decided to examine the question of survival from the shock of thermal injuries. The present report deals solely with results of the latter study.

Materials and methods. Three hundred and thirty-nine (339) male and female white rats of the Wistar strain, weighing between 150 and 298 g, were used in this study. All the animals were anesthetized with ether; their backs, *i.e.*, 32-35% of the body surface, were then immersed for 45 seconds into water heated to 90°C(5). Not knowing what dosage of Piromen might prove effective, several exploratory experiments, not enumerated above, were carried out. A preliminary experimental series is included in Table I as Section I. The results of these experiments provided certain dosage reference points for definitive and confirmatory experiments. The *control animals* consisted of 157 male and female rats of weights comparable to those of the animals treated with Piromen. They received placebo injections of non-pyrogenic distilled water (30 rats), saline solution (10 rats), or the 1/6 molar sodium r-lactate buffer, which is the vehicle of the drug, Piromen (117 rats). The volume of the placebo, 0.1 to 0.2 cc, equalled that of the drug given to the treated animals. The *treated rats* (182), received Piromen in doses of 0.1 μg to 0.6 μg. As shown in Table I, 18 rats received 0.6 μg; 83 rats, 0.2 μg; 15 rats, 0.15 μg; and 55 rats, 0.1 μg of the drug. They are arranged in the Table in 3 groups according to schedules of administration of the drug. The intraperitoneal route of administration of the drug and of the placebo was used, with one exception in which the intravenous route was chosen for comparison (Group K). The time of the initial injection was immediately after inducing the thermal injury in all cases except Group L, in which the initial injection was delayed until 4 hours afterward. All experimental and control animals were segregated into individual cages 12 hours prior to the experiment and were maintained separately for the survival period of 15 days.

Results.† Table I illustrates the results obtained in the present study and shows the marked difference between the Piromen-treated animals and those which received only placebo injections. Under the conditions of the experiments, one can see a dose-weight relationship which was necessary for survival.

In Group A, receiving single daily injections of Piromen at 0.6 μg per rat, it will be noted that no beneficial results were obtained. All of the animals died within 12 hours, ex-

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* Supported by a grant from Baxter Laboratories, Morton Grove, Ill. Results of present experiments were presented in partial fulfillment of the degree, Master of Science, by L. C. Greene.

† For preliminary report, see (6).

TABLE I. Summary of Experiments.

Group	Wt, g	No. of animals	Drug	Dose, μg	Deaths				Survivors at 15 days	Initial wt of survivors, g
					0-4 hr	4-12 hr	12-24 hr	1-14 days		
I. Single daily inj. for 15 days										
A	280-298	16	Pl.		7	8	0	1 (2 days)	0	—
		18	P.	.6	6	12	0	0	0	—
B	263-287	6	Pl.		2	4	0	0	0	—
		9	P.	.2	0	0	0	0	9	263-287
C	185-207	14	Pl.		6	7	1	0	0	—
		14	P.	.1	0	0	0	0	14	185-205
II. Inj. 3 times daily for 15 days										
D	280-290	23	Pl.		4	18	0	1 (2 ")	0	—
		23	P.	.2	0	2	0	0	21	282-287
E*	150-158	10	Pl.		2	8	0	0	0	—
		10	P.	.1	0	0	0	2 (2 ")	8	150-158
	190-220	13	Pl.		4	7	0	2 (7 ")	0	—
		17	P.	.1	0	0	0	3 (2 ")	14	190-218
III. Single inj. immediately after thermal injury										
F	154-210	22	Pl.		4	18	0	0	0	—
		22	P.	.2	5	17	0	0	0	—
	269-298	20	Pl.		6	12	2	0	0	—
		29	P.	.2	1	9	1	0	18	269-287
G	246-278	13	Pl.		4	9	0	0	0	—
		15	P.	.15	0	4	2	0	9	250-260
H	237-248	6	Pl.		1	5	0	0	0	—
		11	P.	.12	0	1	0	0	10	237-248
J	237-248	2	Pl.		1	1	0	0	0	—
		2	P.	.1	0	2	0	0	0	—
	154-170	4	Pl.		2	2	0	0	0	—
		4	P.	.1	0	1	0	0	3	154-170
K	190-196	4	Pl.		3	1	0	0	0	—
		4	P.	.1	0	1	0	0	3	190-192
L†	160-235	4	Pl.		1	2	0	1 (8 ")	0	—
		4	P.	.1	0	0	0	0	4	166-235

P. = Piromen; Pl. = Placebo treated.

* Initial inj. was .1 μg; .2 μg, dose was started 24 hr after thermal injury.

† Piromen admin. 4 hr after thermal injury.

cept one of the controls which lived 2 days. The animals of Group B, receiving 0.2 μg of Piromen, survived for a period of at least 15 days. The same was true of the animals in Group C, receiving 0.1 μg of the drug. It is to be noted, however, that there was a considerable difference in weight between the latter 2 groups. Under *Section II*, it will be noted that the administration of Piromen 3 times daily, instead of once daily, did not materially alter the survival rate. The first of 3 injections was given immediately after thermal injury, the second was given 4 hours later, and the third was given 8½ hours afterward. The initial injection appears to have been the important one, for the total daily amount (0.6 μg Piromen) which was optimum for survival in Group D animals, was equal to the amount of the single injection (0.6 μg) which was too large for survival of the animals in Group A. In Group F, it should be noted that 0.1 μg of Piromen per rat was given 3 times daily for the first day but, on subsequent days, the dosage was increased to 0.2 μg 3 times a day. Other experiments had indicated that a single initial dose of 0.2 μg per rat of this weight group failed to bring about survival. It was noted that after an initial optimum dose of 0.1 μg of Piromen, it made no difference that the dose was increased to 0.2 μg on subsequent days. *Section III* represents a large series of experiments performed to determine the efficacy of different dosages of Piromen in relation to different weights of rats, by single initial administration immediately after thermal injury.

Looking at the present study as a whole, it will be seen that the extent and degree of the

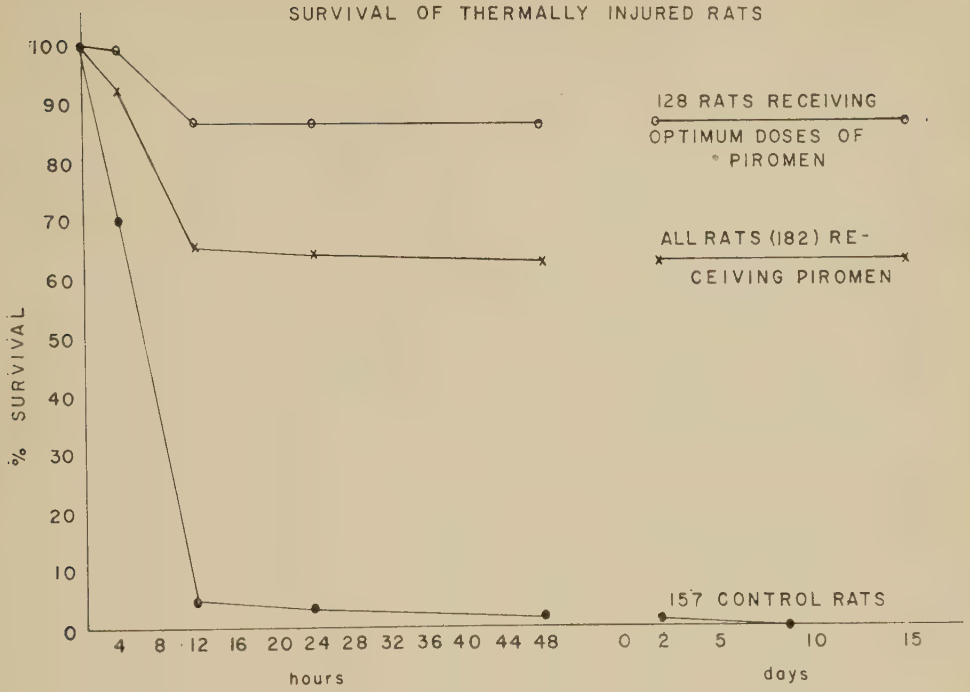

FIG. 1.

thermal injury administered to the placebo-treated animals was 100% lethal in less than the period of 15 days allowed for survival. Approximately 95% of the placebo-treated animals died between 0 and 12 hours. No animal survived longer than 8 days.

The survival of Piromen-treated animals is expressed in Fig. 1. Taking the entire series, irrespective of the dosage of drug administered or route of administration, 62% of the animals survived at least 15 days. Again, the mortality peak occurred between 0 and 12 hours. The experiment was terminated arbitrarily at 15 days, although some animals were not actually killed until 20 days or more after the initiation of the experiment.

The study contains certain experiments in which the size of the dose of the bacterial polysaccharide in relation to the weight of the animal burned was obviously larger or smaller than optimum. Eliminating from calculation certain animals of Group A and parts of Group F and J, and leaving only those to which we consider that an optimum dose of Piromen was administered, the survival rate increases to 86%. Again, the peak of mortality occurred between 0 and 12 hours.

One of the differences between the treated and non-treated animals was that of general prostration which was greatly reduced in the Piromen-treated groups. These animals were more active during the critical 0 to 12 hour period after thermal injury than were the placebo-treated groups.

We are unprepared at the present time to describe in detail the histological changes that occur during the healing of thermal lesions in animals treated with Piromen. A number of sublethal thermal injuries were administered to rats to serve in the histological study. Comparison of these with Piromen-treated animals of the present studies reveal significant differences. The apparent decrease in edema formation, increase in vascularity, and inhibition of scarring in the lesions under the influence of Piromen therapy after sublethal as well as lethal injuries will be discussed in some detail in an article currently in preparation.

Discussion. Death in our experimental animals was associated with shock mechanisms which are not fully understood. We can partially evaluate certain factors which appear to have played a role in the shock, one of which was fluid shift. In previous studies of cats, rats, and guinea pigs, there was a marked tendency towards prevention of the initial edema when Piromen therapy was employed (4). This same apparent inhibition of a fluid shift was associated with survival of the treated animals in the present study. As yet we do not know the nature of the permeability changes effected. When more is known about the role of bacterial polysaccharides in the physiology of connective tissue(7-9) it will be easier to visualize the mechanism by which Piromen influenced the survival rate.

The question of adrenal participation must be considered, since it plays a major role in connection with the shock process. Effects of Piromen on adrenal function have been reported(10-12). The nature of relationship is not entirely clear, but experiments in animals have shown that chronic bilateral adrenalectomy very markedly alters the sequence of events that can be observed when Piromen is administered to an intact animal. Hypophysectomy has been shown to impair the animal's response to Piromen administration much less than adrenalectomy(13). It is tempting to differentiate between a response of connective tissue *per se* to Piromen and a response mediated through or permitted by the adrenal glands. Certain work by Dougherty(14) in eliciting local tissue changes in response to Piromen after adrenalectomy are suggestive of this dualistic action. Whatever the role of the adrenals in response to Piromen administration, it is apparent that the entire pituitary-adrenal mechanism need not be brought into play, and that it is not characteristically a "stress" response.

Analysis of the present data reveals a certain optimum dose-weight relationship. It also suggests that the initial dose of the polysaccharide was the critical one. This optimum dose-weight relationship must be carefully regulated if one is to obtain maximum effects. Calculation from the data in Table I shows an effective range of only 0.5 to 0.75 μg/kg. So far, the dose-weight relationship is applicable only to conditions of the present experiment. It would appear that, as we employ larger, heavier animals and different species of animals, or as we alter the experimental conditions of the thermal injury, such as increasing the area of the body surface involved, a new dose-weight relationship for each particular case may have to be established.

Summary. A bacterial polysaccharide complex, Piromen, was tested for its effectiveness for promoting survival for an arbitrary period of 15 days of rats receiving fatal thermal injuries. An over-all survival of 62% of the rats was observed in this present experiment, regardless of the route of administration, the regimen of administration, and the size of the dose of the drug. No placebo-treated animal survived longer than 8 days and 95% of them died in 12 hours. Results of the present experiments indicate a dose-weight relationship necessary for survival. When such a relationship was recognized and optimum dosage of Piromen was administered to rats, 87% of the treated animals survived.

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A Basis for Comparing the Potency of Gonadotrophins. (20017)

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In an earlier report from this laboratory(1) on inducing ovulation in the diestrous mouse by a single subcutaneous injection of chorionic gonadotrophin, it was suggested that this method might be useful in testing the potency of various gonadotrophins. Such information should be of value in interpreting ovarian responses to these hormones, and should also serve as a basis for comparing the various kinds of units now assigned to gonadotrophins.

To test the validity of this suggestion, bioassays were made of 3 types of commercial gonadotrophins: anterior pituitary extract from the horse,* pregnant mare's serum,† and chorionic gonadotrophin from human pregnancy urine.† Minimal ovulating doses were established for each gonadotrophin.

Procedure. Mature female albino mice of the Rockland strain were injected during diestrus, and the reproductive tracts were examined for evidence of ovulation 18 hours later, as has been explained in several previous reports. During the course of this experiment, it was found that mice weighing at least 20 g yielded more consistent results than mice weighing less than that amount. It is quite possible that the estrous cycle had not yet begun in these lighter animals, even though the vaginal orifice was patent in each. For the purpose of this study, the minimal ovulating unit has been defined as that amount of gonadotrophin which will induce ovulation in 70% of 10 or more diestrous mice. This unit is more reliable for a bioassay of this type than the 50% response formerly suggested in the 1943 report.

TABLE I. Comparison of Capacities of Gonadotrophins to Induce Ovulation.

No. of mice inj.	Wt*	Dosage	% ovulating	No. of ova
		Chorionic gonadotrophin		
17	+	1.4 I.U.	100	1-13
12	+	1.2	83	1-14
9	—	1.2	44	1-5
15	+	1	73	1-16
18	—	1	44	1-7
		Pregnant mare's serum		
12	+	5 I.U.	100	1-18
7	+	4	43	6-15
11	+	3	36	1-9
		Anterior pituitary extract		
14	+	.4 F.H.U.	100	4-14
9	+	.25	88	1-11
12	+	.20	41	1-6

* + = >20 g; — = <20 g.

Results with chorionic gonadotrophin. In preliminary attempts to establish a tentative minimal ovulating dose, mice weighing at least 20 g were injected with either 0.8 I.U. or 0.9 I.U. of the pregnancy urine extract. These amounts proved to be subthreshold doses. 1. *Results with 1.0 I.U.* Eleven (73%) of the 15 mice weighing more than 20 g ovulated. Only 8 (44%) of the 18 mice weighing less than 20 g ovulated. 2. *Results with 1.2 I.U.* Twenty-one animals were used, 9 of which weighed less than 20 g and 12 weighed more than that amount. Of the lighter animals, only 4 (44%) ovulated. Of the 12 mice weighing more than 20 g, ovulation took place in 10 (83%). 3. *Results with 1.4 I.U.* All 17 (100%) of the treated animals weighing more than 20 g ovulated.

Results with pregnant mare's serum. All of the mice used in these tests weighed more than 20 g. None of the animals injected with 2.0 I.U. ovulated. 1. *Results with 3.0 I.U.* Four (36%) of the 11 mice in this series ovulated. 2. *Results with 4.0 I.U.* Seven mice were used in this group, of which only 3 (43%) responded. 3. *Results with 5.0 I.U.* This amount of pregnant mare's serum induced ovulation in all of the 12 mice (100%). Superovulation occurred in 2 animals, 18 ova being found in one and 15 ova in another.

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* Equiphysin, an extract from anterior pituitary of the horse, was kindly furnished by the G. T. Harvey Co., Saratoga Springs, N. Y.

† Generous supplies of Anteron (pregnant mare's serum) and Pranturon (extract from human pregnancy urine) were furnished by Schering Corp., Bloomfield, N. J.

Results with anterior pituitary gonadotrophin. The mice in this series also weighed over 20 g. Numerous animals, not included here, were used to determine an approximate minimal ovulating dose. 1. *Results with 0.2 Fevold-Hisaw Unit (F.H.U.).* Five (41%) of the 12 mice ovulated. 2. *Results with 0.25 F.H.U.* This dosage caused 8 of the 9 animals (88%) to respond. 3. *Results with 0.4 F.H.U.* Ovulation took place in all of the 14 mice (100%).

Discussion. Although the physiological action of both the chorionic gonadotrophin and the pregnant mare's serum is measured in terms of the International Unit, yet the capacity of each to induce ovulation in the diestrous mouse, unit for unit, is certainly not the same. The minimal ovulating unit was found to be only 1 I.U. for the chorionic gonadotrophin, but it was approximately 5 I.U. for the pregnant mare's serum. The minimal ovulating unit of the anterior pituitary extract, measured in terms of Fevold-Hisaw Units, was found to be only 0.25 F.H.U.

Experimenters are using these 3 types of gonadotrophins with 2 distinctly different bases of standardization. Even the chorionic gonadotrophin and the pregnant mare's serum are likely to yield confusing results when used in equal dosages in terms of International Units. Since the main function of the ovary is to produce ova, it would seem logical to assume that a test for the potency of a gonadotrophin should properly be in terms of its capacity to induce ovulation. If mouse-ovulating units are used, then the ovulating potency of the various gonadotrophins will be comparable. Application of these results shows that nearly 5 times as much pregnant mare's serum as chorionic gonadotrophin was necessary to produce equivalent results, and that as little as 0.25 Fevold-Hisaw Unit of the anterior pituitary extract was as potent as the other gonadotrophic substances. This method, which is completed within 18 hours, gives a clear picture of ovarian activity and does not depend upon the interpretation of data gathered after a series of injections. In the latter procedure, the question arises whether the corpora lutea, seen under such conditions, are the result of the first or of a later injection. As has been stressed before, the reaction of the ovary within the first 24 hours after an injection of a gonadotrophin is of great significance in understanding subsequent responses of that organ.

Again we wish to emphasize the importance of using mice weighing at least 20 g. In one series, even more consistent results were obtained by using animals weighing 22 g. That there are major differences in ovarian responses in those mice that weighed 17 g to 19 g and those that weighed at least 20 g is shown by the series of mice injected with chorionic gonadotrophin. In the 1.2 I.U. series, of those weighing at least 20 g, 83% ovulated, but only 54% of the lighter animals responded. Similarly, in the 1.0 I.U. series, ovulation occurred in 73% of the heavier mice, whereas only 44% of the lighter animals ovulated.

Summary. 1. Three types of gonadotrophins were compared on the basis of the capacity of each to induce ovulation 18 hours after a single subcutaneous injection of a minimal ovulating unit of the extract into mature diestrous mice weighing at least 20 g. The minimal ovulating dose is defined as the smallest amount of gonadotrophin which will induce ovulation in 70% of a group of 10 or more mice similarly treated. 2. The minimal ovulating dose for the chorionic gonadotrophin was found to be 1.0 I.U.; for the pregnant mare's serum, approximately 5.0 I.U.; for the extract of the anterior pituitary of the horse, 0.25 Fevold-Hisaw Unit. 3. This bioassay method is of definite value in comparing the potency of various gonadotrophins.

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Effect of Carbohydrates on Aspartic Acid Deaminase Activity of Bacteria.*

(20018)

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The observation that glucose, when incorporated into growth media, exerts an inhibitory effect upon bacterial deaminase activity is established, but the mechanism of action has been obscure(1). Recently evidence was presented in support of the concept that the glucose effect is not primarily on apoenzyme production, but rather the coenzyme of certain bacterial deaminases is resolved during glycolysis(2).

The present paper is concerned with the effects of other carbohydrates and the variation in the mechanism of action in different strains of microorganisms.

Materials and methods. The microorganisms studied were strains of *Escherichia coli, Aerobacter aerogenes, Proteus vulgaris,* and *Bacterium cadaveris.* Two types of media were employed. Medium A consisted of 1% each of yeast extract and pepticase and 0.5% K_2HPO_4 adjusted to a final pH between 6.5 and 7.0. Medium B was composed of 0.1% each of KH_2PO_4, K_2HPO_4, and NaCl; 0.4% $(NH_4)_2SO_4$; 0.07% $MgSO_4 \cdot 7H_2O$; 0.05% sodium citrate; and 0.5% vitamin-free acid-hydrolyzed casein, adjusted to a final pH between 6.5 and 7.0. It was necessary to employ casein in medium B to furnish carbon sources for those control cells which were grown in the absence of a carbohydrate. The carbohydrates were added aseptically from sterile 20% solutions to yield the concentrations desired. The cells were incubated for a period of from 16 to 18 hours at 30°C, harvested by centrifugation, washed once with distilled water, and resuspended in distilled water to give a cell concentration of 0.5 to 1 mg of bacterial nitrogen per ml. The deamination experiments were performed at pH 7 in M/20 phosphate buffer at 37°C, using adequate controls without added aspartic acid. The reaction was terminated by the addition of 0.2 ml of 25% trichloroacetic acid, the tubes centrifuged, and an aliquot of the supernatant fluid removed for ammonia determination by Nesslerization. Color was measured in a Klett-Summerson photoelectric colorimeter using a blue filter (400-465 mμ).

Results. Since previous data on the inhibitory effect of glucose had been obtained by growing bacterial cells in the presence of 2% glucose(3), it was desirable to determine the effect of other concentrations of this sugar on aspartic acid deaminase activity. Flasks of medium B containing from 0.02 to 2% glucose were inoculated with *E. coli,* strain 86 G, incubated for 18 hours, and the aspartate deaminase activity of the washed cell suspensions determined. Fig. 1 presents the results of this experiment. A concentration of 2% glucose was the most effective, whereas 0.02% exhibited essentially no inhibition. Since 0.2% was relatively as effective as 2%, this concentration of glucose was employed in subsequent experiments.

These findings led to studies of the effects of other sugars and related compounds on

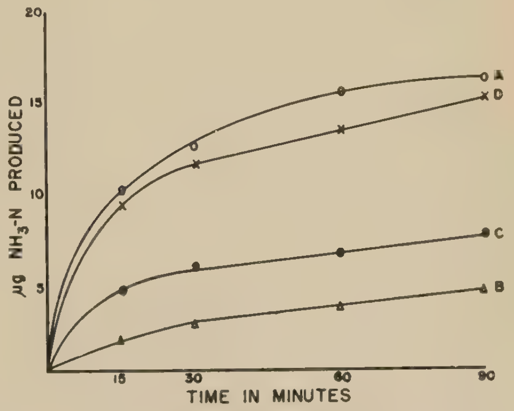

FIG. 1. Effect of graded concentrations of glucose in growth medium A on the aspartic acid deaminase activity of *Escherichia coli* (.085 mg bacterial nitrogen per tube). A = no carbohydrate; B = 2% glucose; C = .2% glucose; D = .02% glucose.

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TABLE I. Effects of Presence of Glucose in Synthetic and Complex Media, as well as of pH, on Aspartic Acid Deaminase Activity of Several Bacteria.

		μg ammonia-nitrogen produced in 60 min.					
		Medium A			Medium B		
Organism	Cell N/tube (mg)	Basal, pH 7 (6.9-7.2)*	Basal, pH 5 (5.2-5.7)	Basal + glucose, pH 7 (5.2-5.9)	Basal, pH 7 (6.3-6.6)	Basal, pH 5 (5.2-5.7)	Basal + glucose, pH 7 (4.8-5.9)
Bact. cadaveris	.07	52.8	33.4	13.9	48.1	38.6	14.8
E. coli (Tenn.)	.085	45	50.5	14.8	53.4	45.8	29.6
(Crookes)	.11	53.7	21.2	62.9	21.8	18.9	49.3
(Gratia)	.2	87	29	89.3			
(Texas)	.085	51	51	23.8	67.6	64.4	21.8
A. aerogenes (Tenn.)	.07	46.4	26.2	4.9	39.2	22	4.6
P. vulgaris	.11	31.3	32.6	16.8	36.5	36	22.3

* Figures in parentheses represent final pH range at time of harvest of cells.

deaminase activity in order to ascertain the specificity of the so-called glucose effect. Representative members of the triose, pentose, hexose, and disaccharide groups were chosen for investigation using *A. aerogenes* as the test organism. The individual carbohydrates were incorporated singly into growth medium A at a concentration of 0.2%. The flasks of media were inoculated with *A. aerogenes,* incubated for 18 hours, and the aspartate deaminase activity of the washed cell suspensions determined. In Exp. 1 (initial pH of media adjusted to 6.5) using 0.085 mg of cell nitrogen per tube, the ammonia produced in 30 minutes was the following: no carbohydrate (final pH 6.5), 14.4 μg NH_3-N; glucose (final pH 4.9), 5.8 μg NH_3-N; xylose (final pH 4.9), 7.8 μg NH_3-N; glycerol (final pH 6.0), 9.9 μg NH_3-N; sucrose (final pH 6.5), 17.4 μg NH_3-N. In Exp. 2 (initial pH of media adjusted to 6.7) using 0.18 mg of cell nitrogen per tube the ammonia produced in 30 minutes was the following: no carbohydrate (final pH 6.7), 62.1 μg NH_3-N; glucose (final pH 4.6), 8.7 μg NH_3-N; citrate (final pH 7.1), 31.3 μg NH_3-N; inositol (final pH 5.3), 29.0 μg NH_3-N; maltose (final pH 5.1), 9.0 μg NH_3-N. It is clear from the changes in pH during growth that this strain of *A. aerogenes* utilized each of the compounds tested save sucrose. It is equally manifest that all of the compounds utilized had an adverse effect on the aspartic acid deaminase activity of the harvested cells, although the degree of inhibition varied considerably among the carbohydrates tested. On the basis of these data it may be concluded that the effect on deaminases attributed to glucose is common to other carbohydrates utilized by the organisms during growth. The results with citrate were particularly revealing for, although this carbohydrate was less effective than others, it did exert a definite inhibitory action on the aspartate deaminase activity of the cells, in spite of the fact that there was an increase in pH during growth rather than a decrease as is noted with the other carbohydrates utilized by this organism.

Even though the foregoing results supported the hypothesis that the adverse effect of the presence of carbohydrate during growth on certain enzyme systems is not due to the resultant drop in pH(1), nevertheless the effect of increased hydrogen ion concentration was reinvestigated and the effect of other medium constituents on the carbohydrate action was determined. Seven strains of microorganisms were employed in order to ascertain whether or not strain variation exists. Medium A and B were used with and without the addition of 0.2% glucose at an initial pH of 7.0. For control purposes, a flask of each medium without added carbohydrate and adjusted to pH 5 was included in order to simulate the increase in hydrogen ion concentration anticipated when the carbohydrate was present.

The results of these experiments (Table I) reveal several points of interest. It is noted that the nature of the medium had little effect on the deaminase activity of the cells or on the inhibitory action of the added carbohydrate.

More significant, however, was the variation in response exhibited by different strains to altered pH and/or the presence of glucose. On the basis of these responses, the 7 strains studied may be divided into 3 groups. Group 1, composed of *E. coli,* strains Tennessee and Texas, and *P. vulgaris,* exhibited no pH effect, while a definite glucose effect was apparent. Group 2, composed of *Bact. cadaveris* and *A. aerogenes,* was affected by both the increased hydrogen ion concentration and by the presence of glucose, but the carbohydrate effect was far greater than that of the altered pH. Group 3, composed of the Gratia and Crookes strains of *E. coli,* exhibited a reduced aspartic acid deaminase activity when grown at acid pH, but no reduction in enzyme activity when grown in the presence of glucose. Growth in the glucose medium was associated with an increase in hydrogen ion concentration.

Discussion. It is manifest from the results presented that the presence of glucose or other fermentable carbohydrates in the growth medium exerts an adverse effect on the aspartic acid deaminase activity of the harvested cells of many, but not all, bacterial species studied. In those strains which are adversely influenced by the presence of glucose the effect is much greater than can be accounted from the resultant increase in hydrogen ion concentration. As noted earlier, the results with citrate speak strongly for an action not at all related to the concurrent pH change. This is also supported by the observation that the 2 strains not affected by the presence of glucose in the growth medium exhibited a lowered deaminase activity when cultured at a pH close to that which resulted from the utilization of the carbohydrate.

It is pertinent here to comment that in sharp contrast to the carbohydrate effects noted in this study and others where the sugar had been incorporated into the growth medium, the addition of such substances to resting cell suspensions resulted in a pronounced stimulation of the rate of deamination of aspartic acid(4).

Summary. 1. The reduction in aspartic acid deaminase activity in cells harvested from a medium containing glucose has been shown not to be specific to this sugar, but rather is shared by other fermentable carbohydrates. 2. The effect of the carbohydrate varies among the several strains studied, and is not associated with the increase in hydrogen ion concentration due to the production of fermentation acids.

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Uric Acid Production in Normal and Gouty Subjects, Determined by N^{15} Labeled Glycine.* (20019)

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By measuring the isotopic composition of urinary uric acid in 2 normal men and one gouty subject after the administration of isotopically labelled (N^{15}) glycine, Stetten and his co-workers(1) were able to demonstrate conclusively that overproduction of uric acid was largely responsible for the hyperuricemia in the gouty subject. They emphasized that because of the expense and labor involved, the study was restricted to a single patient with gout, who was selected on the basis of a large urinary uric acid output and minor tophaceous involvement. Whether the disclosed metabolic defect is seen only in this relatively rare type of case or is manifest in other types and stages of gout could not be answered.

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For this reason it was considered desirable to study the rate of uric acid formation in the more commonly encountered type of disease. Therefore, a gouty subject with a normal uric acid excretion was selected for this study along with a normal individual for the sake of comparison. The experimental procedure of Stetten *et al.* was followed. Both subjects were fed N^{15} labeled glycine in a single dose and were maintained on the same constant diet throughout the period of study. Daily urine samples were analyzed for total nitrogen and uric acid and for isotope abundance in both of these fractions.

Procedures. Subjects. H.S., a white male, 44 years of age, weighing 64.1 kg, had experienced recurrent attacks of gouty arthritis for approximately 4 years. He was unaware of a family history of gout. He had had rheumatic fever as a child and had suffered from intermittent claudication for 2 years. He had mitral stenosis and border-line congestive failure at the time this study was made. A small tophus in the right ear showed the typical urate crystals and gave a positive Murexide test. Nonprotein nitrogen and phenolsulfonphthalein excretion tests were both normal. Serum uric acid varied from 9.0 to 9.3 mg %. Daily urinary uric acid excretion was 474 ± 14 mg. A.F.M., a white male, 30 years of age, weighing 82.0 kg, was apparently free of all disease and gave no family history of gout. His serum uric acid was 3.7 mg %. His daily excretion of uric acid on the same constant diet given patient H.S. was 663 ± 45 mg.

Materials and methods. N^{15} labeled glycine was synthesized from potassium phthalimide which contained approximately 55 atom % N^{15}. The Schoenheimer and Ratner(2) method was used with the following modification: The ethyl phthalimido-acetate was split as indicated, the phthalic acid filtered off, and the filtrate brought to dryness *in vacuo*. The residue was extracted with a small amount of cold water. This solution, which contained the glycine-hydrochloride, was passed over a Duolite A 4 Anion exchanger column (50 to 60 g) instead of treating it with silver carbonate and then with hydrogen sulfide. Before the exchanger column was used it was treated with approximately 2-3 N NaOH and then washed until a neutral pH was obtained. The glycine which was not adsorbed by the column was washed out with small amounts of distilled water (total volume 600 to 800 ml). The water-clear eluate was brought to a very small volume *in vacuo,* and the glycine precipitated with alcohol. The yield was 97%. The elementary analysis for nitrogen checked within the experimental error. The isotope abundance was 55.15 atom % excess. Both subjects were maintained on the same constant, 2000-calorie diet, poor in purine (140 mg) and relatively low in protein (60 g). After 4 days on this diet both subjects were given tagged glycine well mixed with breakfast (100 mg per kg of body weight). The 24-hour urine samples were collected over a few milliliters of concentrated acetic acid and kept at 4°C. All urine specimens were worked up immediately. The total nitrogen was determined on each 24-hour sample by the micro-Kjeldahl method. The uric acid was determined by differential enzymatic spectrophotometry, as outlined by Kalckar(3) and adapted for clinical use by Praetorius(4). The highly purified uricase was prepared by a relatively simple method(5) which followed the general principles outlined by Holmberg (6). Uric acid was isolated from fresh urine according to Talbott *et al.*(7) with minor modifications. The nitrogen content and the ultraviolet analysis for uric acid checked within the experimental error. Isotope abundance of total nitrogen and uric acid was determined according to Sprinson and Ritten-

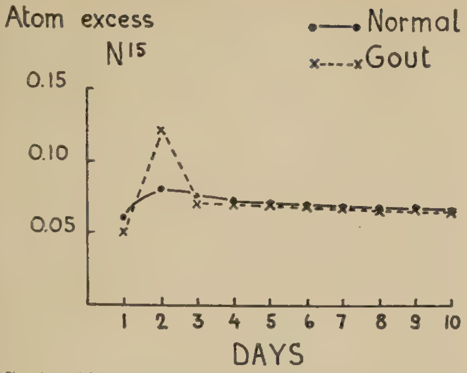

FIG. 1. Abundance of N^{15} in excreted uric acid after ingestion of tagged glycine.

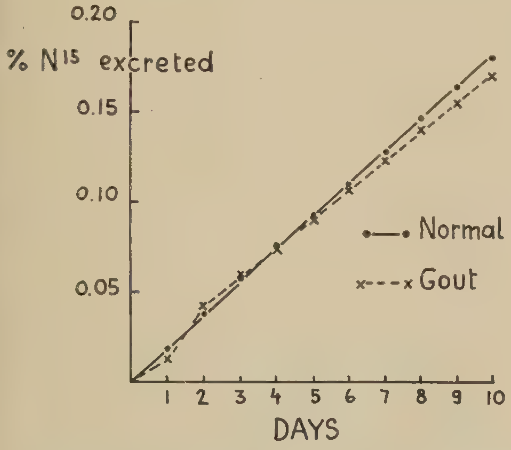

FIG. 2. Cumulative excretion of N^{15} as uric acid expressed as percentage of administered dose.

berg(8).

Results. Total urinary excretion of N^{15} of both subjects was similar to that found by Stetten *et al.* By the 10th day 46.4% of the total dose administered had been excreted by the normal subject and 55.3% by the gouty man.

There was no significant difference in the *urinary uric acid isotope concentrations* of the gouty and the normal subject (Fig. 1). There was, however, a sharp rise in the isotope concentration of the gouty subject between the first and second days and an equally rapid fall on the second and third days. The cumulative excretion of N^{15} in uric acid, expressed as percentage of the total dose administered, was the same in the normal and in the gouty subject (Fig. 2). A comparison of the actual figures revealed excellent agreement between the values on our 2 subjects, gouty and normal, and the 2 normal males reported by Stetten *et al.*

Discussion. This study was designed specifically to determine the production of uric acid from a known purine-precursor, glycine, in a gouty subject with a normal uric acid excretion. For this reason the subject selected was an individual with proven gout characterized by recurrent attacks of gouty arthritis, a hyperuricemia, and minor tophaceous involvement. The control subject was a normal man. The results are quite in contrast to those reported by Stetten *et al.*, in that they show that the actual amount of the precursor incorporated in uric acid is no different in the gouty subject and the normal individual. These experiments, however, do not tell us whether in spite of the same over-all production, the 2 individuals handle glycine the same way qualitatively, *i.e.*, whether they use the same intermediary pathways in purine synthesis. The exact significance of the sharp rise and a subsequent rapid fall in the uric acid isotope concentration in the gouty patient remains unexplained (Fig. 1). The results, however, do show that an increased production of uric acid, at least as far as the incorporation of the nitrogen of dietary glycine in the purine ring is concerned, is not always an essential finding in gout.¶ From these data it cannot be decided whether this implies that patient H.S., in comparison with the case reported by Stetten *et al.*, is simply in a different stage of the disease or displays a different disease mechanism. Additional studies will be required in order to establish whether overproduction of uric acid is an inconstant and nonessential mechanism for the hyperuricemia of gout.

Summary. 1. Glycine was synthesized by the Schoenheimer and Ratner method with a modification (ion exchange resins) which in our hands gave better yields. 2. N^{15} tagged glycine was fed to a normal and to a gouty subject while they were on the same constant, low protein, low purine, 2000-calorie diet. Daily urine samples were analyzed for total nitrogen and uric acid, as well as for isotope concentration in these 2 fractions. 3. No significant difference could be found between the 2 subjects in the isotope distribution of the total nitrogen or uric acid. The percentage of N^{15} excreted as uric acid was no greater in the gouty than in the normal subject. The significance of this finding in relation to the pathogenesis of gout is discussed.

¶ Similar observations have also been made by Stetten *et al.*

We wish to thank Dr. Gordon L. Brownell in whose laboratory the isotope analyses were carried out.

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Inhibition of Thyroid Function by Cortisone and ACTH in Hypophysectomized Rats.* (20020)

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There is experimental and clinical evidence that administration of cortisone or ACTH suppresses thyroid function(1-5). Similar findings have been obtained under experimental conditions which are known to be accompanied by increased secretion of cortical hormone, including injection of epinephrine(6) or formalin(7) and exposure to abnormal temperature(8). Some investigators believe that this inhibition of thyroid function is caused by suppression of secretion of TSH(1,2); however, differences between the inhibition produced by adrenal hormone and by lack of TSH have been pointed out(9,10).

The experiments reported in this paper were undertaken in order to investigate the possibility that the inhibiting action of cortical hormone is exerted at the thyroid rather than at the pituitary level. A similar investigation was reported by Woodbury *et al.*(11).

Material and methods. Hypophysectomized male rats of the Sprague-Dawley strain were divided into 4 groups. Group one served as control. Group 2 was injected for 5 days with either ACTH¶ or cortisone.¶ Group 3 received daily injections of a TSH preparation¶ for 5 days. Group 4 was treated with both TSH and either ACTH or cortisone. The hormone dosage is indicated in the tables. After 4 days of treatment all animals received 4 μc of I^{131} (carrier free)¶ by intraperitoneal injection. Twenty-four hours later the rats were killed; the thyroid glands were dissected free of connective tissue and weighed on a Roller Smith torsion balance. The glands were then macerated in 5% NaOH; aliquots were plancheted and the radioactivity determined by means of a Geiger Counter.

Results. Results of experiments with ACTH are presented in Table I, those with cortisone in Table II. It is evident that the amount

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¶ The TSH preparations were generously supplied by Dr. Daniel McGinty of the Parke Davis Co., ACTH was obtained from Armour and Co. through the courtesy of Dr. E. E. Hays, and Cortisone from Merck and Co. through the courtesy of Dr. E. Alpert. The I^{131} was obtained from the Atomic Energy Commission, Oak Ridge Laboratories.

TABLE I. Influence of ACTH on Uptake of I^{131} in Hypophysectomized Rats.

	No. of rats	% uptake of I^{131} 24 hr after tracer, 4 μc: Total	% uptake of I^{131} 24 hr after tracer, 4 μc: Per mg thyroid wt	Thyroid wt, mg.
Exp. 1				
1. .85% NaCl	7	3.3 ± .53	.51	7
2. ACTH 4 mg/day	4	1.05 ± .19	.1	11.5
3. TSH 1 "	6	29.8 ± 2.8	2.53 ± .20	11.8
4. TSH 1 mg, ACTH 4 mg	5	22.1 ± 1.17	2.07 ± .39	12
Diff. 3-4		7.7 $p<.05$	.46 $p<.3$	
Exp. 2				
1. .85% NaCl	10	.83 ± .22	.09	9.1
2. ACTH 4 mg/day	10	.67 ± .12	.08	9.7
3. TSH 1 "	8	19.8 ± .89	1.84	10.8
4. ACTH 4 mg, TSH 1 mg	9	19.2 ± .64	1.73	11.5
Diff. 3-4		.6 $p<.2$		
5. TSH 2 mg/day	10	27.7 ± 1.81	2.14	13.3
6. ACTH 4 mg, TSH 2 mg	9	24.4 ± 3.19	1.80	13.7
Diff. 5-6		3.3 $p<.4$		
Exp. 3				
1. .85% NaCl	8	.75 ± .23	.08	10
2. ACTH 4 mg	9	.77 ± .16	.08	10.2
3. TSH 1 "	9	22.3 ± 1.57	1.77 ± .15	12.8
4. ACTH 4 mg, TSH 1 mg	9	12 ± 1.34	.95 ± .18	13
Diff. 3-4		10.3 $p = <.01$	.82 $p = <.01$	

of TSH (1 mg or 2 mg) was adequate to increase the uptake of I^{131} significantly. Simultaneous administration of ACTH (Table I) lowered the uptake of I^{131}; in 2 of 3 experiments the difference in uptake values between the TSH treated and the TSH-ACTH treated animals was statistically significant. Similarly, simultaneous treatment with TSH and cortisone significantly lowered the uptake of I^{131} as compared with that of the rats injected with TSH alone (Table II).

Discussion. Observations in intact animals and in man have indicated that endogenous or exogenous cortical hormone suppresses thyroid function. The uptake of a tracer dose of I^{131} by the thyroid gland is diminished following administration of cortisone in the intact rat(4), and in man(1,5). Similar results have been obtained by subjecting rats to various forms of stress(6-8,13,14,16). Migeon *et al.* have found cortisone treatment to diminish I^{131} uptake by the thyroid in adrenalectomized but not in intact rats(12).

In humans, treatment with large doses of cortisone is often followed by clinical and metabolic manifestations of hypothyroidism, such as low BMR(1,2), high serum cholesterol (1,2), and low serum protein-bound iodine (5,15).

It has been assumed by some authors that this inhibition of thyroid function is the result of diminished production and/or release of TSH from the pituitary. This assumption is supported by the observation that administration of exogenous TSH simultaneously with cortisone counteracts the thyroid-depressing effect of the latter(1).

However, in experiments reported by Perry (9) and by Albert *et al.*(10), discharge of thyroidal I^{131} was not influenced by cortisone or ACTH, whereas the uptake of I^{131} was inhibited. Moreover, the increase in cell height due to TSH is not diminished by simultaneous cortisone medication(17). This response pattern differs from that produced by deficient secretion of TSH; in this state both uptake and release of iodine and the thyroid cell height are decreased.

The experiments reported in this paper, and similar work of Woodbury(11) demonstrate

TABLE II. Influence of Cortisone on Uptake of I^{131} in Hypophysectomized Rats.

		% uptake of I^{131} 24 hr after tracer, 4 μc		
	No. of rats	Total	Per mg thyroid wt	Thyroid wt, mg
Exp. 4				
1. Control	9	$1.16 \pm .13$	.12	9.9
2. Cortisone 5 mg/day*	8	$.40 \pm .06$	.04	9.8
3. TSH 1 "	8	$11.42 \pm .64$	.96	12.4
4. Cortisone 5 " * TSH 1 "	9	$9.16 \pm .28$	.88	10.4
Diff. 3-4		2.26 $p = < .01$	.08 $p > .8$	
Exp. 5				
1. Control	9	$.69 \pm .08$	.06	11.3
2. Cortisone 5 mg/day*	10	$.96 \pm .25$	.08	11.8
3. TSH 1 "	10	$19.92 \pm .81$	1.48	13.7
4. Cortisone 5 " * TSH 1 "	11	14.79 ± 1.08	1.17	13.5
Diff. 3-4		5.13 $p = < .01$	.28 $p < .2$	

* In 2 divided doses.

that uptake of I^{131} by the TSH-stimulated thyroid is diminished by administration of cortisone or ACTH to hypophysectomized animals; this phenomenon, therefore, cannot be the result of diminished secretion of TSH; obviously endogenous or exogenous cortical hormone inhibits the thyroid-stimulating action of TSH, as measured by uptake of I^{131}, at extrapituitary levels, perhaps at the thyroid gland level.

In man, clinical and metabolic manifestations of hypothyroidism have been observed after prolonged treatment with cortisone(1,2, 5,15). It is obvious that in such cases not only the uptake of iodine, but also the release of thyroid hormone must be inhibited. The inhibition of I^{131} uptake by cortical hormone, acting at the thyroid level, may be followed, after prolonged administration, by diminished secretion of thyroid hormone. It is possible, too, that the cortical hormone may exert a dual action, directly at extrapituitary levels, and later also through suppression of TSH production. The various reported observations could be reconciled by either of these hypothetical assumptions.

Summary. Administration of cortisone or ACTH decreases the TSH-induced uptake of I^{131} by the thyroid of hypophysectomized rats. This indicates that the suppression of the iodine concentrating function of the thyroid is brought about at extrapituitary levels, and not by suppression of TSH secretion from the pituitary. Some discrepancies of reported effects of adrenal hormones upon thyroid function are discussed.

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Nutrition of *Leptospira canicola.* III. Utilization of Vitamins and Amino Acids.* (20021)

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The present investigation is an extension of the authors' studies on the nutritional requirements of leptospira(1-4). A chemically-defined basal medium developed earlier was shown to be satisfactory for the cultivation of the spirochete when supplemented with dialyzed rabbit serum(3) or an electrophoretically-homogeneous rabbit serum albumin(4). Related studies on the utilization of amino acids and vitamins by pathogenic leptospira have been reported by other workers(5-11).

Experimental. The response of *Leptospira canicola*[‡] to individual amino acids was determined by replacing the amino acid mixture in the previously described basal medium supplemented with rabbit serum albumin(4), with each of 22 amino acids in turn at 0, 10, 20, 40, 60, 80, 100, 150, 200 and 500 mg % concentrations. Seven replicate tubes were prepared at each concentration. The pH of the medium was 7.2-7.4. 0.3 ml of a suspension of *L. canicola,* prepared as described earlier(4), was added to each of 5 tubes with 2 tubes serving as controls. The final volume was 6 ml and the tubes were incubated for 7 days at 32°. Growth was measured as increase in optical density of the cell suspensions determined with the aid of a Klett-Summerson photoelectric colorimeter. In some experiments the inoculum was obtained directly from the 4th or 5th serial transfer of the organism grown on the medium (complete except for the omission of amino acids) in which growth had decreased progressively. The purpose was to determine whether or not the response of the spirochete had been affected by this treatment. Subsequently, an amino acid mixture simulating the composition of rabbit serum albumin was employed in an attempt to replace the albumin and the amino acid mixture of arbitrary composition used initially. The composition (Table I) of the electrophoretically-homogeneous rabbit albumin was determined by assaying for alanine by the method of Sauberlich and Baumann(12) and the 16 other amino acids by the procedures of Dunn *et al.*(13,14). All assays were performed in triplicate using 5 levels of sample and 15 levels of standard. The maximum deviation of the values at the

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[‡] *L. pomona* (strain 9), *L. canicola* (dog Utrecht IV) and *L. icterohaemorrhagiae* (strain 20, Wijnberg Type AB) were obtained through the courtesy of Dr. K. F. Meyer, University of California, San Francisco. *L. ballum* (strain mouse 27), *L. sejroe* (strain Mallersdorf II) and *L. icterohaemorrhagiae* (biotype A, strain Kantorowicz) were obtained through the courtesy of Dr. J. W. Wolff, Institute for Tropical Hygiene, Amsterdam, Holland.

TABLE I. Amino Acid Composition of Rabbit Serum Albumin.

Amino acid	%*	Amino acid	%*
Alanine	4.1	Lysine	8
Arginine	5.3	Methionine	.29
Aspartic acid	7.5	Phenylalanine	11.9
Cystine	5.3	Proline	4.1
Glycine	2.3	Threonine	4.1
Glutamic acid	9.9	Tryptophan	.35
Histidine	3	Tyrosine	6
Isoleucine	3.2	Valine	6.6
Leucine	9.5		

* Determined by microbiological assay using methods described in the text. The rabbit albumin preparation analyzed contained 4.98 mg N/ml. Values are calculated to 16% N.

different levels of sample was 6%. Tests were also made on the effectiveness of asparagine (80 mg %) as a substitute for the amino acid mixture in the medium used to maintain the organism for 8 weeks in serial subculture and to determine which group constituents of the arbitrarily-selected purine-pyrimidine-mineral mixture(3) were essential. In these studies the concentration of each constituent was the same as that employed previously. The response of the organism to 12 vitamins (nicotinamide, nicotinic acid, riboflavin, pyridoxine, pyridoxamine, thiamine, biotin, choline, folic acid, inositol, pantothenic acid and *p*-aminobenzoic acid) was determined by adding each vitamin in turn to a chemically-defined medium less complex than used in the earlier-described experiments. This medium was an aqueous solution of Na_2HPO_4 312, KH_2PO_4 420, NaCl 1280, Na_2CO_3 32, KCl 32, $CaCl_2$ 32, and asparagine 80 (the numbers are mg % concentrations) supplemented with rabbit albumin. Thiamine chloride was tested at 0.001, 0.01, 0.1, 1.0, 10, 100 and 200 γ % concentrations and each other vitamin at 1, 10 and 100 γ % concentrations. Later, the growth response of *L. canicola* in the described basal medium, supplemented with rabbit albumin and 10 γ % of thiamine chloride, was followed for 2 months in weekly serial subcultures. This medium was also tested for its ability to maintain *Leptospira ballum*,[†] *Leptospira icterohaemorrhagiæ* (Biotype A and Strain 20),[†] *Leptospira pomona*,[†] and *Leptospira sejroe*.[†]

Results and discussion. As shown in Table II the growth of *L. canicola* was increased by a supplement of L-arginine • HCl (40-150 mg %), L-asparagine (40-200 mg %), L-aspartic acid (60-100 mg %), L-glutamic acid (40-500 mg %), or proline (10-500 mg %). The data are not shown for the remaining amino acids tested. L-Glutamine was without effect. Sixteen amino acids (Dl-alanine, L-cysteine • HCl, glycine, L-histidine • HCl, DL-isoleucine, L-leucine, DL-lysine • HCl, DL-methionine, Dl-norleucine, DL-norvaline, DL-phenylalanine, DL-serine, DL-threonine, DL-tryptophan, L-tyrosine and DL-valine) were not stimulatory and were definitely inhibitory at some level. It was also found (data not given) that the 5 amino acids (each tested at 40 and 80 mg %) listed in Table II stimulated the growth of *L. canicola* (inocula prepared from the amino acid deficient medium) as much or more on the experimental medium as on the peptone medium containing dialyzed rabbit serum.

Attempts to replace the rabbit serum albumin in the medium with an amino acid mixture simulating the composition of the albumin (Table I) were unsuccessful since concentrations of the amino acid mixture exceeding 10 mg % inhibited the growth of *L. canicola*.

It was observed (data not shown) that the basal medium containing rabbit albumin and asparagine (80 mg %) in place of the amino acid mixture permitted undiminished growth of *L. canicola* when transferred weekly over the experimental period (8 weeks). Although growth was depressed in the absence of the vitamin-purine-pyrimidine-mineral solution, only the vitamins appeared to be essential components of this mixture. It was found, also, that *L. canicola* could be transferred satisfactorily for 6 weeks (experimental period) in media containing glutamic acid (40 mg %), arginine (40 mg %), aspartic acid (80 mg %), or proline (80 mg %) in place of asparagine. *L. icterohaemorrhagiæ* could be maintained on the asparagine medium. The effectiveness of asparagine in replacing peptone in a medium containing serum has been reported by Greene(2) and Stuart (10) and it is of interest that Savino and Renella(9) grew *L. icterohaemorrhagiæ* on a chemically-defined medium containing asparagine.

TABLE II. Response of *Leptospira canicola* to Amino Acids.*

	Increase in optical density									
	Amino acid concentration, mg %									
Amino acid	0	10	20	40	60	80	100	150	200	500
L-Arginine • HCl	41	45	50	53	53	57	55	53	31	8
L-Asparagine	41	48	51	55	51	51	53	55	54	31
L-Aspartic acid	51	53	49	51	57	55	56	0	0	0
L-Glutamic acid	45	48	47	51	49	51	45	45	46	54
L-Proline	27	43	48	49	44	49	47	60	51	51

* Basal medium supplemented with rabbit serum described previously(4). See text for experimental conditions.

TABLE III. Response of *L. canicola* to Vitamins.

	Increase in optical density*			
	Vit. conc., γ %			
Vitamin	0	1	10	100
A				
Nicotinamide	14	13	16	16
Nicotinic acid	14	10	10	14
Riboflavin	14	9	8	14
Pyridoxine • HCl	14	8	12	14
Pyridoxamine • 2HCl	14	9	10	9
Thiamine • Cl	14	42	39	39
Control				
Complete vit. mixture		35		
Stock medium		44		
B				
Biotin	6	15	5	9
Choline • Cl	6	7	8	10
Folic acid	6	11	5	11
Inositol	6	6	7	5
Ca. dl-pantothenate	6	9	8	13
p-Aminobenzoic acid	6	5	10	10
Control				
Complete vit. mixture		32		
Stock medium		48		

* Avg of 4 tubes. A and B not conducted simultaneously. In A the basal medium of asparagine and salts contained 1.5 ml/tube of albumin preparation No. 3 (4.98 mg N/ml). Albumin preparation No. 4 (5.35 mg N/ml; 1 ml/tube) was used in B. Stock medium (Witte's peptone, salts, dialyzed rabbit serum).

The growth-enhancing activity of vitamins (tested singly) is shown in Table III. In further tests (data not shown) thiamine chloride in concentrations from 1 to 200 γ % was found to promote growth of *L. canicola* as effectively as the complete vitamin mixture. The spirochete was subcultured serially for 2 months in the medium containing only the albumin, salts, thiamine and asparagine. The observation that growth was not markedly affected by the omission of asparagine, in disagreement with the results reported earlier(4) showing the need for amino acids in serial subculture, may be explained (possibly) by antagonistic effects of certain B vitamins rather than by any need of the organism for particular amino acids. *L. icterohaemorrhagiæ* (Biotype A), *L. ballum*, *L. pomona*, *L. sejroe* and *L. canicola* were subcultured satisfactorily over the experimental period (14 weeks) in the stock transfer medium (Schuffner's medium with dialyzed rabbit serum) and in a medium composed of thiamine, asparagine, Ringer's solution, Sorensen's buffer and the rabbit albumin. In all cases the growth was good but somewhat less satisfactory in the latter than in the former medium. *L. icterohaemorrhagiæ* (strain 20) could not be subcultured longer than 7 weeks in the second medium. Brede and Lempfrid(15) have reported that 12 species and 23 pathogenic strains of leptospira could be grown in Korthof's medium in which rabbit serum was replaced by allantoic fluid from 13-day-old chick embryos.

It seems probable from the authors' experiments that serum albumin *per se*, rather than an amino acid or collection of amino acids liberated from this protein, is essential for *L. canicola* and other pathogenic leptospira. Although Marshall(6) has suggested that the primary role of the albumin may be to inactivate or adsorb some toxic substance, it seems unlikely from the present experiments that the albumin has this function. Whether the entire protein structure or a particular stereochemical unit is essential for growth of pathogenic leptospira remains to be determined.

Summary. It has been shown that the growth of *Leptospira canicola* on a chemically-defined medium lacking amino acids but supplemented with rabbit serum albumin is stimulated at certain levels of arginine, aspartic acid, glutamic acid or proline but is inhibited

by any one of 16 amino acids at some concentration. The rabbit albumin could not be replaced by a mixture of amino acids simulating the amino acid composition of the albumin determined by microbiological assay. Thiamine was found to be the only vitamin essential for the growth of *L. canicola*. *L. canicola, L. pomona, L. ballum, L. sejroe,* and *L. icterohaemorrhagiæ* could be subcultured on a simple medium containing only salts, thiamine, asparagine, and rabbit serum albumin.

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Physiologic Responses Attending Administration of Antimony, Alone or with Simultaneous Injections of Thyroxin.* (20022)

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Brady, Cowie, and also Maren and Otto have reported the high localization of antimony in the thyroid gland of the dog(1-3). Apparently this localization is not species specific since similar results were obtained by Smith(4) in the hamster, and by Kramer(5) in the rabbit. In this laboratory a strong affinity for antimony was demonstrated by the thyroids of rats exposed to the inhalation of finely divided antimony trioxide, and by rats fed a low level of antimony trioxide in their diet(6). The growth curve for the latter group of animals showed a slightly greater increase in body weight than the controls. The increase in body weight and the sluggish behavior of these animals, accompanied by the marked accumulation of antimony in the thyroid, were interpreted as a tendency toward hypothyroidism. If antimony did have antithyroidal activity when administered alone, one would expect that when given with simultaneous injections of thyroxin, their antagonistic actions should result in a nearly normal metabolic state.

In view of the above facts the following experiments were designed to determine the effect on thyroid function of antimony administered alone or in combination with thyroxin. Three experiments were conducted, 2 using albino rats and one using albino rabbits. The body weight and oxygen consumption were employed as measures of thyroid activity. The concentration of antimony in the tissues was determined in order to ascertain whether or not the presence of thyroxin influenced the pattern of distribution of antimony in the body.

Methods. Oxygen consumption was deter-

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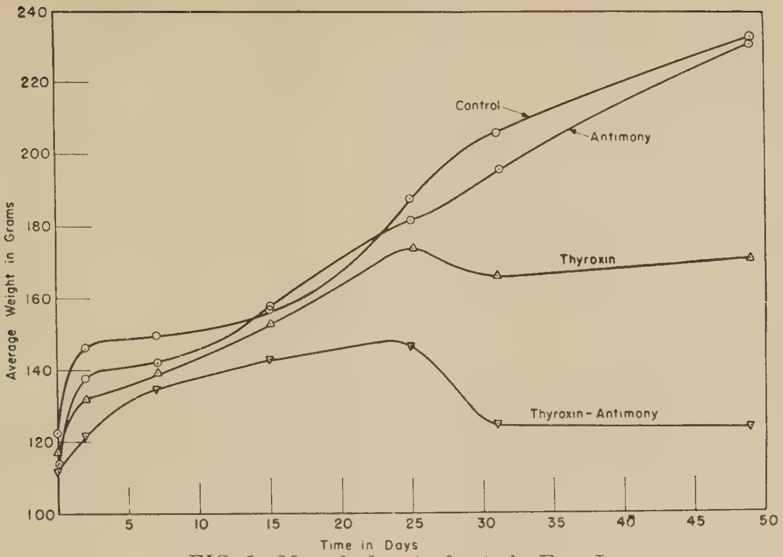

FIG. 1. Mean body wt of rats in Exp. I.

mined by using the apparatus devised by D'Amour and Blood(7). The animals were deprived of food for 12 hours prior to performing the test. Inasmuch as metabolism studies may be influenced by the time of day, the temperature, the amount of noise and activity in the animal room, an effort was made to keep these conditions relatively constant in the experiments. Maren's method for the microdetermination of antimony in the presence of organic material was used(8). In those cases where excessive amounts of iron interfered with the procedure (*e.g.*, liver and spleen) it was necessary to follow Freedman's modification(9) of Maren's method.

Procedure. The male albino rats of the Sprague-Dawley strain used in Exp. I and II were approximately 5 weeks of age at the beginning of the experiment. Each rat received 15 g of synthetic casein diet daily, previously determined as adequate for the normal growth and development of the control animals. *Exp. I.* Twenty rats were divided into 4 groups of 5, as follows: A. Control group; B. Thyroxin-treated group; C. Thyroxin-treated antimony trioxide group; D. Antimony-trioxide group. The animals in Groups B and C received subcutaneous injections of thyroxin daily, the dosage being 0.1 mg/100 g body weight. The antimony dosage in Groups C and D was 2% antimony trioxide added to the casein diet. The rats were weighed periodically. The oxygen consumption was determined after one, 2, 3, 4, and 6 weeks of treatment. At the end of 49 days the rats were anesthetized with ether and bled by cardiac puncture. Blocks of organs were removed for histologic study and preserved in 10% formalin. The concentration of antimony was determined in specimens from Groups C and D. *Exp. II.* Twenty-four rats were divided into 4 groups of 6, as in Exp. I. This study was the same as the preceding one with regard to the concentration of thyroxin and the level of antimony, but the oxygen consumption was not determined until the 54th day. The experiment was terminated after 56 days following the same procedure used in Exp. I. *Exp. III.* Eight full-grown male albino rabbits of approximately the same age and body weight were paired into 4 groups on the basis of treatment as in the previous experiments. The rabbits were fed *ad libitum* on Purina stock diet. The antimony trioxide was force fed in gelatin capsules to insure a daily dose of 13 mg/kg of body weight. The thyroxin was injected subcutaneously, the daily dose being 0.33 mg/kg body weight. The oxygen uptake was measured on the 20th day. The animals were sacrificed by air embolism on the 21st day. Specimens from Groups C and D were analyzed for antimony.

TABLE I. Changes in Oxygen Consumption (cc O_2/100 g Body Wt/Min.) over a 6-Wk Period.†

Time on test, wk	O_2 consumption							
	Control		Thyroxin		Thyroxin-Antimony		Antimony	
	(Mean	S.D.)	(Mean	S.D.)	(Mean	S.D.)	(Mean	S.D.)
1	2.74	.34	3.21	.37	3.13	.35	3.24	.12
2	2.46	.16	2.95	.55	2.90	.37	2.53	.21
3	1.75	.17	3.14*	.49	3.26*	.33	1.72	.18
4	2.41	.33	4.61*	.51	6.12*	.56	2.62	.29
6	2.15	.16	4.38*	.22	5.37*	.42	2.54	.37

* Denotes statistically significant difference when compared with the control value ("p" = .01).

† Five rats in each group.

TABLE II. Mean Body Weights and Oxygen Consumption Values (on the 54th Day) of Rats in Exp. II.†

Group	Mean body wt (g)		Oxygen consumption, cc O_2/100 g body wt/min.	
	Initial	Final	(Mean	S.D.)
Control	123.6	185.6	2.72	.27
Thyroxin	121	127	3.71*	.17
Thyroxin-antimony	118.8	112.8	4.89*	.31
Antimony	122.6	192.1	1.98*	.31

* Denotes statistically significant difference when compared with the control value ("p" = .01).

† Six rats in each group.

TABLE III. Initial Weight of Rabbits, Weight of Animals on Autopsy and Oxygen Consumption Values Obtained after 20 Days of Treatment.

Group	Rabbit No.	Body wt (kg)		Oxygen consumption (cc O_2/kg body wt/min.)
		Initial	Final	
Control	1	2.1	2.3	5.95
	2	2.3	2.3	5.62
Thyroxin	1	3.2	2.4	10.25
	2	3.1	2.5	6.51
Thyroxin-antimony	1	3.3	2.4	17.52
	2	3.2	2.4	16.84
Antimony	1	2.8	3	4.59
	2	2.7	2.7	5.75

Results. The results of the growth studies of Exp. I are presented in Fig. 1. The average weight of the antimony group parallels that of the control group. The thyroxin-antimony group showed only a slight gain in weight during the first 3 weeks and suffered a drastic loss of weight in the fourth week. The oxygen consumption studies (Table I) indicate that the oxygen uptake of the antimony group did not vary significantly from that of the control group during the 6-week period. In the third week thyroxin exerted comparable effects on the oxygen consumption of the thyroxin and thyroxin-antimony groups, but a greater increase in oxygen consumption was observed in the thyroxin-antimony group in the latter half of the experiment.

The results of Exp. II are set forth in Table II. The average body weights of these animals were of the same order as those of Exp. I. The oxygen consumption values are similar to those of the preceding experiment. That is, in the order of increasing oxygen uptake the groups are: antimony, control, thyroxin, and thyroxin-antimony.

The results of Exp. III (Table III) indicate that the rabbit was similarly affected by the treatments. The metabolism of the antimony group, as reflected in the body weight and oxygen uptake, did not differ from the control group. The hypermetabolic condition of the thyroxin-antimony group was more pronounced than that of the thyroxin group.

In view of the metabolic changes brought about by the combined effects of thyroxin and antimony, the relative distribution of antimony in the tissues of the thyroxin-antimony group was compared with that of the antimony group (Table IV). The mean antimony concentration for each organ of the thyroxin-antimony group, except the adrenal, exceeds that of the antimony group. The results are not statistically valid, however, because of the wide individual variations within the sample.

The findings of the chemical analyses of the rabbit tissues yielded a distribution pattern of antimony in the tissues very similar to that determined in the rat, although the concentrations were lower because of fewer doses.

TABLE IV. Distribution of Antimony in Rat Tissues. 8 rats.

	Organ, concentration of antimony (γ/g)						
	Liver	Kidney	Heart	Spleen	Lung	Adrenal	Thyroid
				Thyroxin-antimony			
	10.6	6.4	12.1	52.9	—	—	117.6
	7.8	4.1	8.6	8.9	—	51.1	111.6
	6.3	8.9	13.2	11.1	—	56	313.7
	13.2	5.6	5.9	15.9	9.8	52.3	35.5
	14.4	9.6	8.1	17.7	14.3	14.5	17.2
	23.7	16.7	17.5	39.9	26.4	52.4	57.7
	14.4	11	13	12.4	12.8	30	—
	21.4	19.7	16.2	15.4	24.9	90	33.4
Avg	14	10.3	11.8	21.8	17.6	49.5	98.1
				Antimony			
	2.1	4.7	4.9	4.6	—	—	30
	3	3.5	4.8	7.6	—	87.5	10.7
	3.6	3.9	10.2	27.6	—	44.3	28.7
	16.4	10.2	10.9	26.8	18.6	96.6	280
	16.5	7.7	1.2	24.3	10	52.7	118.4
	7.8	5.9	4.2	20.4	12.5	—	106.6
	23.2	10	12.7	—	28.4	50	154.5
	1.9	6.9	5.8	20.7	10.1	76	47.3
	5.7	6.3	12.5	—	4.7	—	23.5
Avg	8.9	6.7	7.6	18.9	14	67.8	88.9

The histologic differences found in the thyroids of the thyroxin and the thyroxin-antimony groups did not vary significantly from those observed in the glands of the control groups during phases of the normal secretory cycle. The thyroids of the antimony group displayed some hyperplasia.

Discussion. Tissue respiration has been shown to be inhibited by antimony in the kidney(10) and in the liver(11). Chen *et al.* (12), and Barron and Kalnitsky(13) have further demonstrated that antimony interferes with cellular metabolism by combining with the sulfhydryl group of the respiratory enzymes. On the basis of these observations one would expect a general depression of metabolism following antimony administration. Kramer(5), however, recently reported that antimony did not affect the metabolic rate as reflected by the oxygen uptake and serum cholesterol level. The results of Exp. I and III confirm Kramer's observation that antimony does not influence oxygen consumption. The diminished oxygen uptake of the antimony group in Exp. II appears to be atypical in that further studies in progress substantiated the results obtained in Exp. I and III.

The results obtained in the thyroxin-antimony groups do not support the original hypothesis, namely, that thyroxin and antimony have antagonistic actions. It appears that antimony enhances the action of thyroxin resulting in a hypermetabolic condition which is more severe than that produced by thyroxin alone. The apparent absence of histologic change in the thyroid gland of animals given thyroxin and antimony suggests that the mode of action of the test materials in this group may be extrathyroidal, whereas the action of antimony alone appears to be within the gland in the nature of a thyroid block.

The results of the chemical analyses indicate that antimony is widely distributed throughout the tissues. With the exception of the thyroid concentration the results are not completely in agreement with those reported previously(1,2,4,5,14-16). The route of administration, the level of dosage and the number of doses are factors which may have influenced the relative distribution in the tissues. In addition to the thyroid, the adrenal showed a strong affinity for antimony as also did the lung and spleen. The accumulation of antimony in the latter 2 organs may be explained by the hypothesis that the reticulo-endothelial cells are involved in the removal of antimony from the system.

Summary. A study was made on the metabolic effects and the tissue distribution of antimony when administered alone or with simultaneous injections of thyroxin. It was found that: 1) there appeared to be little effect on the body weight or oxygen uptake following ingestion of antimony trioxide in the amounts employed in these studies; 2) thyroxin activity was intensified by antimony trioxide resulting in a greater degree of hypermetabolism than was observed with thyroxin alone; 3) the relative distribution of antimony in the tissues was not significantly altered by injections of thyroxin; 4) the glandular hyperplasia observed in the thyroids of the antimony group was not present in the thyroxin-antimony group which suggests a difference in the mechanism of action.

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Influence of Insulin on Uptake of Monosaccharides by the Isolated Rat Diaphragm. (20023)

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The mechanism whereby insulin produces an increase in the phosphorylation of glucose is unknown. The effect of insulin may be due to some direct action on the hexokinase system or to some action on the permeability of the cell which makes more substrate available for phosphorylation. The latter hypothesis was developed by Levine *et al.*(1) who demonstrated that the volume of body fluid throughout which d-galactose is distributed in the eviscerated, nephrectomized dog is increased after the administration of insulin. Since galactose is neither utilized nor excreted by the eviscerated dog, Levine postulated that insulin exerts its effect by facilitating the rate at which the hexose enters the cell. Subsequently Levine and his colleagues(2) reported that insulin exerts a similar effect on other sugars having the same configuration as d-glucose in the first 3 C atoms.

In order to test further the hypothesis that insulin exerts an action on the transfer of sugars across the cell membrane, the effect of insulin on the *in vitro* uptake of several hexoses and pentoses by the isolated rat diaphragm was studied. The sugars used were highly purified preparations of d-glucose, d-galactose, d-fructose, d-xylose, d-arabinose, and l-arabinose.

Method. Male rats of the Wistar strain, weighing between 150 and 200 g were fasted overnight and killed by stunning and decapitation. In each experiment the 4 hemidiaphragms from 2 rats were placed in 5 ml of a chilled solution of phosphate-saline(3) containing 0.2% of the particular sugar being studied. The tissues were shaken in the cold solutions for 5-7 minutes, blotted gently on filter paper, and transferred to Warburg vessels in such a manner that each flask contained a left hemidiaphragm from one rat and a right hemidiaphragm from the other rat. The incubation mixture consisted of 2 ml of the same solution of sugar in buffer as was used in the pre-equilibration period, but one flask of each pair contained amorphous insulin in addition. The concentration of insulin in the incubation mixture was 0.02 unit per ml when glucose was present, while 0.1 unit per ml was used with the other sugars. The vessels were maintained at 37°C and shaken at a rate of 116 cycles per minute in an atmosphere of oxygen. Generally an experiment at each of 4 different incubation periods, 30, 60, 120 and 240 minutes, was performed each day. At the end of the incubation period the tissues were blotted and weighed on a torsion balance. The average wet weight of tissue in each flask was 250 mg. The *uptake of each sugar* was calculated by subtracting its final concentration, after removal of the tissue, from that of a portion of the medium incubated simultaneously without tissue. For determination of glucose, fructose, and xylose the Nelson method was used(4); with galactose and the 2 isomers of arabinose the method was modified slightly in that the time of heating with the copper reagent was increased to 40 minutes. The standard solutions always consisted of the sugar being studied. In about half of the experiments on the uptake of fructose the results obtained with the Nelson method were confirmed by parallel determinations with the more specific method of Roe(5).

Results. Sugars that are utilized, that is, transformed to other substances and removed as such from the metabolic pool will be removed from the extracellular medium so long

TABLE I. Effect of Insulin on the Uptake of Monosaccharides by Isolated Rat Diaphragm.

Sugar	30 min.			60 min.			120 min.			240 min.		
	Uptake, mg/g	Insulin effect, mg/g	P	Uptake, mg/g	Insulin effect, mg/g	P	Uptake, mg/g	Insulin effect, mg/g	P	Uptake, mg/g	Insulin effect, mg/g	P
d-glucose	1.09 ± .26* (6)	+.82 ± .18	<.01	1.84 ± .09 (12)	+1.55 ± .14	<.001	3.01 ± .15 (6)	+1.54 ± .29	<.01	5.35 ± .35 (5)	+1.99 ± .44	<.02
d-fructose	—	—		1 ± .28 (6)	+ .55 ± .25	>.05	1.74 ± .14 (10)	+ .75 ± .15	<.001	2.58 ± .37 (10)	+1.84 ± .40	<.01
d-galactose	.67 ± .14 (8)	+.33 ± .09	<.01	.74 ± .11 (8)	+ .44 ± .10	<.01	1.26 ± .21 (8)	+ .11 ± .09	>.2	1.30 ± .12 (8)	+ .23 ± .16	>.1
d-xylose	.64 ± .04 (6)	+.11 ± .12	>.4	.89 ± .31 (8)	+ .22 ± .26	>.4	1.02 ± .20 (6)	+ .10 ± .13	>.4	1.44 ± .24 (6)	+ .31 ± .14	>.05
d-arabinose	.29 ± .12 (6)	+.14 ± .08	>.1	.68 ± .29 (6)	— .10 ± .17	>.5	.76 ± .20 (6)	+ .21 ± .21	>.3	.72 ± .27 (6)	— .24 ± .27	>.4
l-arabinose	.83 ± .26 (6)	+.08 ± .34	>.8	1.10 ± .19 (6)	— .10 ± .34	>.7	.99 ± .17 (6)	+ .63 ± .26	<.05	1.71 ± .32 (6)	+ .23 ± .31	>.4

* Mean ± S.D.

Numerals in parentheses represent No. of experiments.

as the enzymatic systems involved in such transformations remain intact. Sugars which are merely transported across the membrane into the cell will be removed from the medium until they can no longer be stored as such.

If it be assumed that the extracellular compartment of muscle is about 10% of the wet weight(6) and that this compartment reaches equilibrium with the incubation medium during the 5 minutes of pre-equilibration, then the maximum uptake that can be attributed to diffusion in 2.0 ml of a medium with a sugar concentration of 200 mg % is given by the expression: $(2)\ (.9) \left(\frac{2}{2 + .9\ (\text{wet wt})} \right)$. Accordingly, with a wet weight of 0.25 g, the maximal uptake by the muscle as a result of diffusion from the medium will be 1.6 mg per g.

The data summarized in Table I reveal that the isolated rat diaphragm removes appreciable quantities of all sugars studied. The uptake of d-galactose, d-xylose, and l-arabinose approaches, as an asymptote by 4 hours, the value attributable to maximal diffusion of the sugars into the intracellular compartment. The uptake of d-arabinose, however, is maximal at 2 hours and is about half that of the other pentoses; the difference between the mean total uptakes of the 2 isomers of arabinose by 4 hours is highly significant (P 0.01).

The effect of insulin on the uptake of glucose by the muscle is apparent at all incubation periods. A statistically significant effect of insulin on the uptake of d-fructose is apparent only after one hour of incubation. With d-galactose, a significant increase in uptake is produced by insulin after 30 and 60 minutes of incubation but not thereafter. With l-arabinose, the effect of insulin is significant after 2 hours incubation but not thereafter. With the other sugars, no statistically significant effect of insulin could be demonstrated at any period of incubation. It is pertinent to note that some of the positive effects of insulin would have been missed if only one or another incubation period were utilized.

Discussion. The data reported herein reveal that d-galactose, d-xylose, and l-arabinose are taken up by the diaphragm at a rate which can be attributed to diffusion of the sugars into the cell. Whereas the uptake of d-arabinose is about half that of the other pentoses, the uptake of d-glucose and d-fructose exceeds that attributable to maximal diffusion. The contrast between the uptake of d-arabinose and that of the other non-utilizable sugars suggests that the terminal molecular configuration may determine whether or not the sugar can continue to enter the cell until its intracellular concentration reaches that of the extracellular medium.

In accord with the postulate of Levine and his colleagues, insulin enhances the uptake of d-galactose and l-arabinose. In disagreement, however, is the observation that insulin does not influence appreciably the uptake of d-xylose and increases markedly the uptake of d-fructose.

Consequently, the effect of insulin cannot be attributed solely to the proximal molecular configuration of the sugar. The data, however, support the hypothesis that insulin acts on some process, other than the hexokinase reaction, which facilitates the rate of which some sugars enter the cell.

Summary. The uptake of d-galactose, d-xylose, and l-arabinose by the isolated rat diaphragm is such as can be attributed to equilibration with extracellular fluid. The uptake of d-glucose and d-fructose exceeds that attributable to diffusion while the uptake of d-arabinose does not approach that of the other pentoses. Insulin enhances the uptake of d-glucose, d-fructose, d-galactose, and l-arabinose. The data support the hypothesis that insulin acts on some mechanism other than the hexokinase reaction.

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Acid-Soluble Phosphates in Monkey Brain as Affected by Certain Central Nervous System Stimulants and Depressants.* (20024)

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A number of investigators(1-7) have examined the effects of certain central nervous system stimulants and depressants on the acid-soluble phosphates of brain. In the majority of instances significant changes in inorganic phosphate, phosphocreatine, and adenosine triphosphate (ATP) have been reported to occur. However, there is little uniformity as to the direction these changes take, and in some cases the significance of the alterations might be questioned on the basis of technic or lack of sufficient data. In none of these studies was a concerted effort made to compare the effects of stimulation to those of depression of the central nervous system on the distribution of acid-soluble phosphates within it and in only one investigation(7) were experiments performed on animals higher in the phylogenetic scale than the dog. For these reasons the study described herein was undertaken in an attempt to determine whether or not direction of change of any acid-soluble phosphates in the brain could be correlated with an increase or decrease in activity of the central nervous system.

Methods. Thirty-one adult, male and female, rhesus monkeys were employed in these experiments. Under pentobarbital anesthesia a longitudinal, midline incision was made in the scalp of each animal and the major portion of the calvarium removed. The skin incision was closed loosely and 24 hours later, when the animal had recovered from the anesthetic, the incision was reopened and the brain exposed for subsequent freezing with liquid air. Nine animals received no drug prior to freezing of the brain. Four animals received *d*-tubocurarine hydrochloride (1-2 units/kg) intravenously. When necessary, respiration was maintained artificially until the brain was frozen. The remaining 18 monkeys were divided into 2 groups of 9 each, one group receiving, by the intravenous route, a central nervous system depressant [pentobarbital (35 mg/kg) or chloral hydrate (125 mg/kg)] and the other group receiving a central nervous system stimulant [pentylenetetrazol (20 mg/kg) or 5-ethyl-5-(1,3-dimethylbutyl) barbituric acid(8) (5 mg/kg)]. Immediately after the development of anesthesia or convulsions the cerebrum was frozen by pouring liquid air over the exposed brain. The entire cerebrum was then removed and analyzed for acid-soluble phosphates by the method described by Umbreit *et al.*(9).

Results. Since most of the conclusions in the literature relative to the effects of various stimulants or depressants on the distribution of acid-soluble phosphates in the central nervous system were based on comparisons with control animals which had received a curariform agent, it was of interest to determine the effects of curarization. It would appear, on the basis of the data in Table I, that administration of *d*-tubocurarine produces a rise in inorganic phosphate and a fall in phosphocreatine in the cerebrum. However, when analyzed statistically, only the difference in phosphocreatine content is significant. The mean value for ATP content in curarized animals is not significantly higher than that found in the non-curarized monkey. In animals anesthetized with pentobarbital or chloral hydrate the cerebral content of inorganic phosphate, phosphocreatine, or ATP was not significantly different from those values found in non-curarized controls. Likewise, convulsions, produced by pentylenetetrazol or the convulsant barbiturate, 5-ethyl-5-(1,3 dimethylbutyl) barbituric acid, caused no statistically significant alteration of inorganic phosphate or phosphocreatine content of cerebrum. The ATP content of cerebrum was not altered by pentylenetetrazol convulsions

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TABLE I. Effect of Various Drugs on True Inorganic Phosphate, Phosphocreatine and Adenosine Triphosphate Content of Monkey Cerebrum.

		μM phosphorus/100 g brain (mean ± S.E.)		
Drug	No. of animals	True inorganic phosphate	Phospho-creatine	Adenosine triphosphate
0	9	451 ± 26	155 ± 7	54 ± 16
d-Tubocurarine	4	536 ± 37	98 ± 13	92 ± 24
Pentobarbital	5	466 ± 27	144 ± 11	30 ± 8
Chloral hydrate	4	495 ± 21	146 ± 15	41 ± 6
Pentylenetetrazol	4	515 ± 30	220 ± 31	72 ± 18
5-Ethyl-5-(1,3-dimethyl-butyl) barbituric acid	5	483 ± 25	151 ± 16	*48 ± 22

* Mean not statistically significant.

and it was impossible to determine the significance of the difference of cerebral ATP content between those animals receiving the convulsant barbiturate and the controls, since the mean value for the former was not statistically significant.

Discussion. The data presented in this paper are, in general, not in agreement with the results of similar experiments reported in the literature. This lack of conformity may be due, in some instances, to a difference in the species of experimental animals employed (2,4). In other cases, conclusions have been drawn from inadequately controlled experiments(1) or based on data from a limited number of experimental animals(3,5,6), the number of animals employed being too small to allow a statistical analysis of results for determination of the significance of any differences observed. A single study(7) of the effects of convulsions on the distribution of acid-soluble phosphates in the brain of the monkey has been reported. This study involved one animal convulsed by electric shock which caused a decrease in ATP and phosphocreatine and a rise in inorganic phosphate. The significance of such findings, based on observations on only one animal, are open to question.

Summary. The influence of *d*-tubocurarine, two central nervous system depressants (pentobarbital and chloral hydrate) and two central nervous system stimulants (pentylenetetrazol and 5-ethyl-5-(1,3 dimethylbutyl) barbituric acid) on the concentrations of inorganic phosphate, phosphocreatine, and ATP in the cerebrum of the rhesus monkey has been investigated. *d*-Tubocurarine was the only one of these compounds to produce any statistically significant effect. With this agent there was a significant decrease in phosphocreatine content without any alteration in inorganic phosphate or ATP.

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Lack of Influence of Vitamin B_{12} on Protein Catabolic Action of Cortisone.* (20025)

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The influence of vit. B_{12} on growth and N balance has been the subject of a number of studies. The growth of rats is enhanced by treatment with vit. B_{12} if the animals have free access to food(1). The fact that in rats fed a constant diet, vit. B_{12} fails to influence weight gain or N retention, suggests that the growth-enhancing effect in *ad lib.* fed animals is due to increased food intake and not to changes in the utilization and metabolism of foodstuffs(2). Similarly, in partially starved rats treatment with vit. B_{12} has no effect upon weight loss or N excretion(3). In *ad lib.* fed rats treatment with vit. B_{12} diminishes or prevents the growth retardation and weight loss caused by administration of thyroid hormone(4,5). If the food intake is kept constant, weight loss induced by thyroxine is not influenced by vit. B_{12}; however, under these conditions a significant improvement of N retention is found in spite of the uninfluenced weight loss(2). The growth retarding effect of cortisone is ameliorated by vit. B_{12} in *ad lib.* fed animals(6,7). In view of the fact that vit. B_{12} was found to decrease the N loss in thyroxine treated force fed rats, it was considered of interest to determine whether the vitamin would exert a similar effect upon N loss induced by cortisone.

Methods. Male rats, descendants of the Wistar strain, weighing between 180 and 250 g were used. All animals were tube fed a mixed diet described by Ingle *et al.*(8) twice daily. The animals were gradually adapted to tube feedings, after which they were fed a constant amount during the control and experimental periods. The amount of diet selected for each animal was sufficient to maintain weight or to permit a slight weight gain during the control period. The animals were kept in wire screened, individual metabolism cages in a constant temperature room (22-23°C). The 24-hour urine specimens were analyzed by a micro-Kjeldahl method. The urinary nitrogen excretion and weight of each animal were determined daily for a control period of 9 days, followed by an experimental period of equal length. During the experimental period 9 animals were treated with 3 μg of vit. B_{12} and 2.5 mg of cortisone acetate twice daily; 10 animals received 2.5 mg of cortisone acetate twice daily. All injections were given at the same time each day.

Results. Administration of 5 mg of cortisone daily caused the urinary N excretion to increase by 71 mg over that of the control period (Table I). The increase in the animals receiving 5 mg of cortisone and 6 μg of vit. B_{12} daily, was 64 mg. The difference is minimal, and statistically not significant. Nor did the weight loss of the animals receiving cortisone differ from that of the animals treated with vit. B_{12} in addition to cortisone.

Discussion. The weight loss and the increase in urinary nitrogen excretion induced by cortisone were not influenced by vit. B_{12} when the food intake was constant. This observation differs from that reported by Meites and Ogle(6), who showed that vit. B_{12} decreases, in part, the growth retarding effects of cortisone. The difference of the results of the two experiments is probably due to the different experimental conditions used. In our experiment the food intake was kept constant, and cortisone was administered for a short period, whereas in the experiment of Meites and Ogle the animals had free access to food, and were treated with cortisone for a longer period of time. The appetite as well as the food intake are known to increase in animals treated with vit. B_{12}(6). This appetite stimulating effect of vit. B_{12} might well account for the inhibition of the growth retarding effects of cortisone in *ad lib.* fed animals.

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TABLE I. Effect of Vit. B_{12} on N Excretion of Cortisone Treated Rats Force Fed Constant Rations. N excretion/24 hr.

Treatment (twice daily)	Control period,* mg	Treatment period,* mg	Diff., mg	"p" of diff.	Wt change, g/9 days
Cortisone, 2.5 mg	201	298	+97	.001	–13
	193	265	+72	"	–12
	197	269	+72	"	–19
	203	263	+60	"	–16
	201	277	+76	"	–18
	184	270	+86	"	–17
	181	255	+74	"	– 8
	151	224	+73	"	– 9
	184	238	+54	"	–10
	196	242	+46	"	– 7
Mean	189	260	71		–12.9
Cortisone, 2.5 mg + Vit. B_{12}, .3 mg	204	249	+45	.001	–10
	176	226	+50	"	– 8
	187	220	+33	"	– 8
	186	258	+72	"	–11
	202	280	+78	"	–15
	203	261	+58	"	– 8
	204	282	+78	"	– 8
	205	281	+76	"	–15
	210	292	+82	"	– 8
Mean	197	261	64		– 9

* Mean of 9 days.

Whereas vit. B_{12} does not decrease the nitrogen loss induced by cortisone, the same dose of the vitamin was shown in previous experiments to decrease the nitrogen loss induced by thyroxine(2). The reason for the difference of action of the vitamin on the nitrogen loss induced by cortisone, and by thyroxine respectively, is not known. Possibly the two hormones induce N loss by a different mechanism. The latter is but poorly understood in either instance; it has been suggested that the action of cortisone may be "anti-anabolic" rather than catabolic.

Summary. In rats receiving a constant food intake by tube feeding, the weight loss and N loss induced by cortisone is not improved by treatment with vit. B_{12}. This failure of vit. B_{12} to influence cortisone induced protein catabolism is in contradistinction to the N sparing effect of vit. B_{12} in thyroxine induced protein catabolism.

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Differentiation as a Determinant of the Reaction of Rat Embryos to X-Irradiation.* (20026)

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Two previous papers(1,2) have dealt with the effects of irradiating rat embryos on the 9th and 10th days of gestation. The principal effects in both instances were slowing of the embryonic growth rate, the appearance of developmental malformations and an increase in intrauterine mortality. After a given effective dosage on either day the rate of mortality was generally similar, but growth retardation and the number of malformations were always greater following 9th-day treatment. Thus, on the whole, the rat embryo appeared to be more sensitive to irradiation on the 9th than on the 10th day of gestation. The greater reactivity of the 9th-day embryo was thought to be related to the lesser degree of embryonic differentiation at this age.

The present experiment is an extension of these studies to include the effects of x-irradiation on the 8th day of gestation. Embryos of this age show no differentiation; they consist merely of a homogeneous mass of randomly arranged blastomeres at the embryonic pole of the egg cylinder. This is distinctly more primitive than the condition seen on the 9th day when the embryo is a laminated plate composed of the 3 primary germ layers, ectoderm, mesoderm, and endoderm; and it is in even greater contrast to the 10-day embryo in which actual organogenesis is beginning in several systems.

The *method* was the same as that employed in the previous experiments and only the essential features need be mentioned here. On the 8th day of pregnancy female rats were anesthetized with Nembutal, an incision made in the ventral abdominal wall, and one uterine horn brought to the surface of the incision. Lead plates were so arranged as to shield the mother and all but 2 to 5 of the implantation sites. The unshielded sites were exposed to 12½, 25, 50, 100 or 200r of x-rays† after which the uterine horn was replaced within the abdominal cavity. Before the incision was closed, the number and position of all irradiated and non-irradiated embryos were noted on a diagram to facilitate later identification. Pregnancy was allowed to continue for 3, 5, or 7 days post-irradiation before the embryos were removed, fixed, weighed, and prepared for histologic study. The distribution of irradiated embryos as to dosage, post-irradiation interval, and number of non-irradiated siblings is presented in Table I.

Observations. The rate of intrauterine death among embryos irradiated on the 8th day was not increased above that of their non-irradiated siblings after doses of 12½ to 100r. Exposure to 200r, however, killed 14 of 15 embryos within 3 days and caused severe growth retardation in the survivor. During the same period only one of 24 non-irradiated siblings from the same pregnancies died. A dose of 200r, therefore, was regarded as rapidly lethal and no effort was made to obtain survivors after longer post-irradiation intervals. This dosage has been shown to be lethal also to embryos exposed on the 9th and 10th days (1,2). A difference, however, was noted in the reaction of 9- and 10-day embryos to doses less than 200 r. They showed an appreciable elevation of the mortality rate above control values after exposure to only 100r, and the 9-day embryos exhibited a moderate increase after as little as 50r. Thus, although embryos at the 8th, 9th or 10th days of gestation were equally susceptible to the lethal

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† The beam was that produced by the factors: 85 kvp, 10 ma, and 1.0 mm aluminum added filter. Further characteristics were: HVL of 2.0 mm of aluminum, and the 1.0-cm depth dose exactly equivalent to the air dose. The target-object distance varied between 10 and 20 cm and the duration of exposure never exceeded 2 minutes.

TABLE I. Distribution of Irradiated Embryos as to Dosage, Post-Irradiation Interval, and Number of Non-Irradiated Siblings.

Post-irradiation interval, days	No. of irradiated and control embryos in each dosage group									
	12½r		25r		50r		100r		200r	
	I*	C	I	C	I	C	I	C	I	C
3									15	24
5	9	10	13	15	16	18	10	10		
7	4	3	14	19	12	14	17	13		

* I = Irradiated; C = Control.

TABLE II. Retardation of Growth in Surviving, Irradiated Embryos Expressed as

$$\frac{\text{Mean Wt of Control—Mean Wt of Irrad.}}{\text{Mean Wt of Control}} = \%\ \text{Retardation.}$$

Age when exposed, days	Post-irrad. interval, days	% retardation following			
		12.5r	25r	50r	100r
8	5	1	12	18	30
8	7	5	6	5	14
9*	4		6	10	17
9*	6		1	1	17
10*	3			0	6
10*	4			0	9
10*	5			0	14

* Data taken from previous papers(1,2).

TABLE III. Representative Malformations Produced by Irradiation with 100r.

Day of irradiation	Organ or region affected	Incidence, %
8	None	0
9	Eyes	90
	Brain	45
	Aortic arches	27
	Heart	27
	Spinal cord	18
	Situs inversus	18
	Urinary tract	5
10	Eyes	75
	Urinary tract	25
	Brain	11

action of 200r, those treated on the 8th day were resistant to the lethal effects of smaller doses, whereas those treated on the 9th or 10th days showed a gradation of response.

The growth-retarding effects of irradiation, on the other hand, were more pronounced after treatment on the 8th day than at the older ages. Embryos receiving only 12½r on the 8th day showed a small deficiency in weight when compared with their non-irradiated controls 5 or 7 days later, and the disparity of weights became more striking after higher doses (Table II). Comparable data from previous experiments(1,2), although variable and not strictly equivalent in regard to the post-irradiation intervals, indicate that growth was inhibited to a lesser degree by irradiation on the 9th or 10th days.

No developmental malformations resulted from treatment on the 8th day. This is in sharp contrast to the results of 9th- or 10th-day exposure, after which the occurrence of congenital malformations was one of the most striking effects of irradiation (Table III). Although there was no evidence of aberrant developmental processes following treatment on the 8th day, general retardation in development was apparent in many embryos 5 to 7 days after exposure to 50 or 100r (Table II).

Discussion. These results demonstrate that the reaction of rat embryos irradiated on the 8th day of gestation was more limited than that of embryos exposed under similar conditions on the 9th and 10th days. After treatment at either of the older stages the general pattern of response was similar: the rate of mortality, the amount of growth retardation, and the incidence of malformations increased as the dosage increased—the only major difference being in the degree of response(1,2). After treatment on the 8th day, however, no malformations occurred and the mortality rate did not show a gradation as the dosage increased. This seems to constitute a different pattern of response.

Such a difference can best be accounted for in terms of differing susceptibilities to irradiation of the cells making up the embryos at the respective ages. The susceptibility of cells to irradiation is, in general, considered to be inversely related to the degree of cellular differentiation at the time of irradiation; *i.e.*, the greater the susceptibility the less the differentiation, although exceptions to this generalization are well known. Indeed, the more limited response of the 8th- as compared with either the 9th- or 10th-day embryos seems to represent such an exception, whereas the greater sensitivity of the 9th- as compared with the 10th-day embryo is in accordance with the generalization. Then, in the present case, the diversity of response cannot be explained simply in terms of greater or lesser degrees of embryonic differentiation at the time of irradiation.

It is generally agreed that rapidly proliferating cells such as comprised these embryos may show any of 3 biological reactions to irradiation: 1) death may ensue without further cell division, 2) mitosis may be temporarily inhibited but later resumed at an approximately normal rate, or 3) subtle genic alterations (somatic mutations) may occur, but not be manifest until later in the faulty differentiation of descendant cells. The thresholds of sensitivity of these 3 reactions need not necessarily remain in a set ratio, nor need they change at the same rate, as differentiation progresses. The change in the embryonic response observed here can be explained by postulating that as differentiation began the component cells changed their propensity to show one or the other of the cellular reactions enumerated above. For example, the only observed effects of irradiation on the 8th day was smallness of size, until the dosage level was raised to 200r, at which level total mortality occurred. The simplest interpretation is that all cells which were "hit" or affected in any manner soon died. The death of a moderate number of these totally undifferentiated blastomeres would result in nothing more than a setback in the developmental schedule equivalent to the time required to replace the missing cells. This alone, or perhaps in combination with some mitotic delay in the surviving cells, could account for the retardation observed on the 5th and 7th post-irradiation days. Such destruction of blastomeres presumably could be tolerated up to a critical number, with no consequence other than generalized growth retardation; but destruction beyond this hypothetical point might be expected to cause embryonic death. The absence of an increase in mortality after all smaller doses and the total mortality after 200r are results compatible with such an explanation.

By the 9th day the embryo has already attained the first stage in differentiation, namely, the formation of the primary germ layers. Very likely some of the affected cells in the 9-day embryos, as in the 8-day embryos, were promptly killed by the irradiation; but there is strong evidence that other affected cells in the 9-day embryos reacted in a different way: they survived and continued to proliferate more or less normally until some critical stage in organ formation was reached. The malformations, many of which did not become apparent until several days after irradiation, undoubtedly included some that developed from surviving cells bearing altered developmental potentialities, that is, cells in which the genic make-up had been changed by the radiation. Malformations can result from the deletion of some or all of the cells already induced to form a particular organ, but this mechanism implies specific deficiency of tissues or parts in the abnormal organ. Several of the observed malformations(2) did not involve such deficiencies but, instead, were judged to be the result of aberrant developmental processes. Then, it is probable that certain of the cells that survived irradiation on the 9th day suffered genic alterations which became apparent only later in defective organ formation. It is also possible that some of these somatic mutations had lethal effects and thus secondarily contributed to the increased mortality among the irradiated animals.

On the 10th day differentiation had progressed to the point that overt organogenesis was present in several systems. The fact that embryos of this age reacted in a similar but less pronounced manner than did 9-day embryos, may be taken as an indication that the susceptibility of the cells to the various radiation-induced effects was also similar but that the threshold of reactivity to all such effects had risen. This proportionate lessening in all of the effects is thought to be related simply to the greater differentiation within the

10-day embryo.

The observations presented here warrant the conclusion that the pattern of response to irradiation in embryonic rats undergoes a qualitative change when cellular differentiation begins within the embryo. A similar change in the pattern of response appears to have occurred in a series of irradiated mouse embryos studied by Russell(3), if due allowance is made for the relatively faster developmental schedule of the mouse. The data presented by Job, Leibold, and Fitzmaurice(4), on the irradiation of rat embryos over a wide range of ages, lack sufficient detail to support a positive conclusion, although the possibility of a change in the response to irradiation between the 8th and 9th days of gestation is not contradicted by their results.

Summary. X-irradiation of rat embryos on the 8th day of gestation had a more limited effect on subsequent development than did similar treatment on the 9th or 10th days. The only residual effect after doses of 12½ to 100r on the 8th day was retardation of growth. Exposure to comparable doses on the 9th and 10th days has been shown previously to result in the development of malformations and in an increase in mortality, as well as in retardation of growth. This difference in reactivity appears to be dependent upon whether cellular differentiation within the embryos has begun at the time of irradiation.

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Equal Effectiveness of L and D-Ethionine in Producing Tissue Damage in Rats and Mice. (20027)

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L-ethionine has been found to be as effective in producing fatty changes in the liver of female rats as the racemic dl-ethionine(1). Dl-ethionine produces renal changes in the rat similar to that produced by dl-serine(2). The nephrotoxic action of serine, however, is only caused by the d-isomer and not by the naturally occurring l-serine(3). It seemed therefore of interest to test both the l- and d-isomer of ethionine as to their possible nephrotoxic action as well as to their effect on the pancreas. In addition the influence of both isomers upon the liver and pancreas of male albino mice was tested. Changes induced by ethionine in the albino mouse will be briefly described since this species is apparently well suited for studies of this kind.

Material and method. The L- and D-ethionine were obtained from the California Foundation for Biochemical Research in Los Angeles. The L-ethionine had a specific rotation of $[\alpha]^{25}_{D} = +23.5°$ as a .85% solution in 0.2 normal HCl. The D-ethionine had a specific rotation of $[\alpha]^{25}_{D} = -23.6°$ as a .8% solution in .2 normal HCl. Dl-ethionine was obtained from the Nutritional Biochemical Corp., Cleveland, Ohio. Young male albino rats of the Wistar strain were given a protein free diet as previously described(4). After having been on this diet for 8 days, they received 1 mg ethionine per g body weight in 3 divided doses via the intraperitoneal route. The animals were killed 48 hours later. Male albino mice of the Swiss strain were fed a synthetic diet containing 16% casein. This diet was supplemented with either .5% of L- or D-ethionine. The animals were killed after 5 days. Total liver lipids of some of these mice as well as of controls were measured gravimetrically after extraction with chloroform. In order to study the influence of ethionine on the mouse, young albino mice of

TABLE I. Results of Intraperitoneal Inj. of 1 mg/g Body Wt of L- or D-Ethionine on Pancreas and Kidney of Male Rats on Protein Depletion Diet, Sacrificed after 48 Hr.

			Pancreas		
No. of animals	Administered	Renal necrosis	Loss of basophilia	Swelling of zymogen granules	Necrosis
8	l-ethionine	+++	+	++	+
7	d-ethionine	+++	+	++	+

TABLE II. Influence of Supplementation of a Synthetic Diet Containing 16% Casein with .5% L- and D-Ethionine 5 Days on Pancreas and Liver Including Total Liver Lipids in Male Mice.*

			Pancreas			Liver		
No. of animals	Administered	Renal necrosis	Loss of basophilia	Swelling of zymogen granules	Necrosis	Liver damage	Histologic fat	Total liver lipids,* % (wet wt) ± stand. dev.
6	—	0	0	0	0	0	0	6.85 ± .23
12	l-ethionine	0	+	++	+	+	+++	30.33 ± 2.32
11	d-ethionine	0	+	++	+	+	+++	34.15 ± 1.58

* 4 animals in each group.

the Swiss strain weighing 20 to 30 g were given the casein diet supplemented with .5% dl-ethionine. Animals were sacrificed after 5 and after 28 days. In order to study the maximum effect of chronic ethionine feeding a group of 60 male and 60 female mice were allowed to live for longer periods of time. Of these animals only 8 survived between 50 and 61 days.

Results. As can be seen from Table I there was no difference in the nephrotoxic action of L- and D-ethionine in the rat. The changes observed were essentially similar to those previously described with dl-ethionine(2). There was also no appreciable difference in the effect of both isomers on the pancreas. A comparable degree of pancreatic damage was also seen in mice after they had been given a casein diet supplemented with either L- or D-ethionine for 5 days. The changes in the liver were likewise identical in both groups, and comparable to those found when the racemic dl-ethionine was fed. As can be seen from Table II there was a highly significant increase of total liver lipids in the mice fed either L- or D-ethionine.

The pancreatic changes in the mouse are similar to those previously described in the rat(4-6). There was no significant difference between male and female animals. In the pancreas of mice killed after 5 days there was loss of characteristic basophilia at the base of cells composing the acini and marked swelling of the cytoplasm as well as focal vacuolization and liquefaction necrosis. There was also focal infiltration of inflammatory cells. In the pancreas of some animals after 28 days and in all animals after 50 to 61 days almost complete disappearance of the normal acinar cells containing zymogen granules was found. There were, however, many collapsed acini composed of cells whose nuclei were surrounded by a narrow layer of undifferentiated cytoplasm. There was in addition some fibroblastic proliferation and infiltration by lymphocytes and plasma cells. The islet tissue appeared normal.

The livers of both male and female animals sacrificed after 5 days were markedly enlarged and yellow in color. Microscopically the liver cells were distended by innumerable small droplets which stained intensely with Sudan IV. Focal areas of degenerating liver cells often surrounded by areas of hemorrhage were found irregularly distributed within the liver lobules. After 28 days and even more distinctly in the animals surviving 50 to 61 days the liver architecture was found to be conspicuously distorted on microscopic examination. Single liver cells as well as groups of liver cells were separated from each other by bands of proliferating fibroblasts. In their vicinity a network of reticulum fibers were clearly identified with appropriate staining.

Many of the liver cells showed vacuolization due to infiltration by fat. Some liver cells contained giant nuclei with irregular chromatin particles and from one to several nucleoli.

Fatty infiltration of the proximal convoluted tubules was observed in the kidneys being more pronounced in animals killed after 5 days than in those examined after longer periods. Necrosis of the distal portion of the proximal convoluted tubules was not seen. Since this change has been previously noticed predominantly in rats on a protein deficient diet following the intraperitoneal injection of 1 mg ethionine per g of body weight a similar experiment was carried out with mice. However, renal necrosis was not observed in either male or female animals.

Discussion. The results of these experiments as well as those of Jensen and his coworkers(1) demonstrate clearly that both isomers of ethionine are equally effective in their tissue damaging action. Ethionine was found to have an inhibitory effect on the uptake of methionine into tissue proteins of rats and mice(7). Ethionine is apparently incorporated into tissue proteins with the subsequent formation of abnormal proteins(8). Both optical isomers of methionine can be utilized in various species including the rat and mouse(9). It is now demonstrated that both optical isomers of the methionine antagonist ethionine, are equally effective at least under the conditions tested.

The identical effect of both isomers on the rat kidney is in distinct contrast to that of serine which is limited to the d-isomer. Another difference has been previously noted, namely that the nephrotoxic effect of serine but not that of ethionine can be prevented by the simultaneous administration of several amino-acids for example alanine(10). It should, however, be noted that the nephrotoxic effect of ethionine has so far only been observed in the rat but not in the mouse, dog, cat and monkey(11).

Hepatic and pancreatic lesions can be produced in the mouse by ethionine and thus the mouse may prove to be another suitable species for experiments of this type. It should be pointed out that in the mouse, fatty changes in the liver are considerably more pronounced than in the rat. Severe fatty infiltration is found in both male and female mice after having been on an ethionine supplemented diet for 5 days. Following chronic ethionine feeding, fatty infiltration is almost absent in the liver of rats with intralobular fibrosis(12) while considerable amounts of stainable fat are found in liver cells of mice with ethionine induced intralobular fibrosis. In additional experiments these fatty changes although somewhat less marked were also found in mice fed an ethionine supplemented complete Rockland mouse diet containing 21% protein, instead of the synthetic casein diet. There exists, therefore, in the mouse, a distinct similarity between liver changes produced by ethionine and those induced by diets deficient in lipotropic factors.

Summary. The L- and D-isomers of ethionine are equally effective in producing pancreatic and renal changes in the rat as well as fatty infiltration of the liver and pancreatic damage in the albino mouse. Chronic ethionine administration produces intralobular hepatic fibrosis which is preceded by severe fatty infiltration in both male and female mice.

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Effect of Cortisone on Metabolism of Certain "B-Vitamins" in the Rat.*

(20028)

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In addition to the well-known influence of cortisone on protein metabolism and gluconeogenesis, several components of animal tissues, including blood lipids, cholesterol, and uric acid, have been shown to be increased by administration of this hormone, possibly as a consequence of its action on proteins and carbohydrates. Recently, Kochakian (see Dorfman and Goldsmith)(1) has reported that cortisone causes an increase in liver and kidney arginase in the rat, and Umbreit (see Dorfman and Goldsmith)(1) observed that the decreased concentrations of D-amino acid oxidase in the liver and of proline oxidase in the kidney of adrenalectomized rats could be restored to normal by the administration of cortisone. Microbiological activity of cortisone acetate was reported by Gaines, Broquist, and Williams(2) who found that folic acid in the medium of *Streptococcus faecalis* and citrovorum factor in the medium of *Leuconostoc citrovorum* could be replaced by cortisone. In addition, this steroid was found capable of reversing the inhibition of aminopterin for the latter organism. Mild acid treatment was reported to increase the activity of clinical cortisone (Merck's "Cortone") for *L. citrovorum.*

TABLE I. Composition of Experimental Diet.

Ingredient	Concentration, %
Cerelose	66.05
Casein ("vitamin-free")	18
Mineral mixture	4
Corn oil	5
Sulfathalidine	2
Methocel	4
Choline dry-mix (25%)	.40
Vitamin pre-mix*	.50
Vit A and D concentrate (100000 A, 10000 D)	.05
Alpha-tocopherol acetate	12 mg

* To provide the following quantities of vitamins (μg/g of diet): thiamine 2.5, riboflavin 2.5, nicotinic acid 5, pyridoxine 2.5, calcium pantothenate 10, biotin .05, folic acid .50 and 2-methyl-1, 4-naphtho-quinone .50.

In view of the effect of cortisone on protein and carbohydrate metabolism and on certain enzyme systems, consideration of a possible influence on the metabolism of certain vitamins and on dietary requirements for these factors appeared to be indicated. Meites(3,4) has indicated that cortisone administration increased the vit. B_{12} requirement of the young rat. The present study was undertaken to determine the effect of cortisone on the over-all metabolism of 2 other B vitamins, pyridoxine and riboflavin, as indicated by their urinary excretion by rats, and, in the case of the former, by its influence on survival of rats on a pyridoxine-deficient diet. Additional observations were made on the effect of acid-treated cortisone on aminopterin toxicity, and data were also obtained on blood cell distribution and prothrombin times of rats receiving cortisone.

Methods. Two groups (1 and 2) of 8 male Sprague-Dawley rats each, averaging 54 g were pair-fed through a preliminary period of 3 days and a test period of 7 days on a diet whose composition is given in Table I. This diet contained 2.5 μg of riboflavin and pyridoxine per g, levels calculated to provide adequate, but not excessive, amounts of these vitamins. Prior to being placed on experiment, the rats were fed the diet *ad libitum* for a period of 5 days, following which they were placed in metabolism cages for purposes of urine collection. After the 3-day preliminary period, group 1 was given 2.5 mg cortisone (Merck's "Cortone") per day by intraperitoneal injection and group 2 was given

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* Thiamine hydrochloride, riboflavin, nicotinic acid, pyridoxine, calcium pantothenate, and cortisone (Merck's "Cortone") were generously supplied by Merck and Company, Inc., Rahway, N. J., through the courtesy of Dr. D. F. Green. Folic acid and aminopterin were generously supplied by the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y., through the courtesy of Dr. T. H. Jukes.

TABLE II. Excretion of Nitrogen, Pyridoxine and Riboflavin by Cortisone-Treated Rats and Pair-Fed Controls.

				24 hr excretion of:					
	Avg wt (g)		Avg food consumed (g)	Nitrogen (mg)		Pyridoxine (μg)		Riboflavin (μg)	
Group	1	2	1 & 2	1	2	1	2	1	2
Cortisone treatment	+	—		+	—	+	—	+	—
Preliminary period (days):									
1	53.8	54	6	61.4	53	.51	.46		
2			7	54.4	48.2	.54	.62		
3	70.8	72	8	52.6	48.9	.63	.66		
Experimental period (days):									
1	70.8	72	9	91.4	51.1	.52	.59	4.7	5.1
2			9.1	123.3	81.4	.57	.56	6.7	6.4
3			9.1	117.3	74.9	.69	.76	5.1	4.9
4			8.6	120	83.7	.83	.64		
5			7.7	137.8	86.7	.71	.59		
6			7	125.4	83.3	.68	.71	4.6	4.5
7	75.1	87.8	2.5	106.2	76.6	.71	.76		
Avg (exp. period)				117.3	76.8	.67	.66	5.3	5.2

one ml injection of saline. Urine samples, diluted to 100 ml, were stored under refrigeration at pH 2.0 until assayed. Pyridoxine was determined according to the yeast assay first proposed by Atkin, Schultz, Williams, and Frey(5) using *Saccharomyces carlsbergensis,* after hydrolysis of the samples for 2 hours at 20 lb pressure (pH 2.0). Riboflavin was estimated by the *Lactobacillus casei* method of Snell and Strong(6) and nitrogen was determined by the micro-Kjeldahl method. *Two additional groups* (3 and 4) of 5 male weanling rats each were fed *ad libitum* the diet shown in Table I with pyridoxine omitted. After a depletion period of 49 days, group 3 was given cortisone (2.5 mg per day intraperitoneally) and group 4, injections of saline. Observations on the survival times of the rats in the respective groups were recorded. A *fifth and sixth group* of 8 pair-fed weanling rats were fed the complete diet with daily injections of aminopterin (20 μg). In addition, group 5 received injections of acid-treated cortisone at a level of 3 mg per day. The acid treatment was the same as that described by Gaines *et al.*(2) which resulted in increased citrovorum factor replacement activity for *L. citrovorum.*

Results and discussion. From the data given in Table II for Groups 1 and 2 it is evident that the administration of cortisone had no appreciable effect upon the urinary excretion of either pyridoxine or riboflavin. A characteristic increase in the excretion of nitrogen was observed, along with an inhibition of growth and food consumption. The increase in nitrogen excretion persisted throughout the 7-day period in the case of 5 rats, apparently disappeared after 3 to 4 days in 2 others, and was evident for only one day in the last. Food consumption was limited by the appetite of the cortisone-treated member in 7 of the 8 pairs.

Group 3, given daily injections of cortisone after 7 weeks on a pyridoxine-deficient diet, survived an average of 33.5 additional days on the deficient regimen. Control group 4, receiving saline, averaged 38.7 days survival. Standard deviations were 7.4 days and 9.5 days, respectively. Average daily food consumption was 3.8 g for Group 3 and 4.4 for Group 4. These results are compatible with those on excretion of pyridoxine by cortisone-treated rats, both indicating that the hormone has little, if any, effect on the pyridoxine requirement.

The method of Huff and Perlzweig(7) was employed in an attempt to determine 4-pyridoxic acid in the urine, but this procedure was found to be too insensitive to provide reliable data on the content of this metabolite in the diluted urine samples.

Eight male weanling rats (Group 5) receiving the diet shown in Table I, plus 20 μg of aminopterin per day by injection, survived an average of 7.7 days. Comparable animals (Group 6) receiving, in addition to the same amount of aminopterin, 3 mg per day of acid-treated cortisone, exhibited a mean survival time of 7.3 days. Thus, the reported activity of acid-treated cortisone in reversing the inhibition of aminopterin for *L. citrovorum* does not extend to the rat, at least at the levels used in this experiment.

Eosinopenia was observed in the blood of 7 of the 8 rats of Group 1 after they had received cortisone injections for 7 days. A thromboplastin preparation obtained fresh from acetone-extracted rabbit brain was used in prothrombin time determinations on the animals of Groups 1 and 2. No appreciable differences were noted in prothrombin times between those animals which had received cortisone for 7 days and their controls, the averages being 22 seconds and 21 seconds, respectively.

In view of the observations recorded in the literature, for example, Czaczkes and Guggenheim(8) and Riesen, Schweigert, and Elvehjem(9) that the riboflavin excretion is associated with the level of protein intake, it was surprising that the increased nitrogen excretion by the cortisone-treated rats was not accompanied by a concomitant increase in riboflavin excretion. As a further check, 2 additional groups of 8 rats each were pair-fed the diet shown in Table I for one week, during which time one group received injections of 2.5 mg of cortisone daily and the other received a mock injection of saline. The livers of these animals were then removed and examined for their riboflavin content by the *L. casei* method. In terms of μg per g dry matter, the average riboflavin concentrations in the livers of cortisone-treated and control rats were 72.5 and 66.2, respectively, with standard deviations of 14.8 and 15.5. These results confirm the data on riboflavin excretion and indicate that cortisone had no appreciable influence on the metabolism of this vitamin.

Summary and conclusions. 1. The effect of cortisone administration on the metabolism of certain "B-vitamins" by the growing rat has been studied. This hormone did not influence the excretion of pyridoxine in the urine or the survival time of rats on a pyridoxine-deficient diet. Likewise, no effect was observed on the excretion of riboflavin or on the riboflavin content of the liver, although a marked increase in nitrogen excretion was recorded. Acid-treated cortisone exhibited no activity in counteracting the toxicity of aminopterin. 2. Eosinopenia was observed in rats receiving cortisone, but no differences in prothrombin times were found after 7 days of cortisone administration.

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Pancreatic Hyperglycemic Factor and Growth.* (20029)

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Highly purified preparations of pituitary growth hormone have either a diabetogenic or a growth-stimulating effect depending on the type of experimental animal used(1). The role of the pancreas in the action of this hormone has received extensive study. There is some evidence that growth hormone suppresses insulin secretion(2), while other observations point to a stimulation(1,3). However, growth hormone affects the blood sugar level(4) and nitrogen excretion(5) under conditions in which changes in insulin secretion can not occur. There are a number of observations which suggest that the hyperglycemic factor from pancreas (HGF) is a true hormone originating in either the alpha or delta cells of the islets and that it is involved in the action of growth hormone. This substance has been isolated free from insulin activity from the pancreas of several species and has a specific action on liver glycogen which is demonstrable both *in vivo* and *in vitro*(6,7). It is present in normal amounts in the pancreas of the alloxan-diabetic rabbit and in increased amounts per unit weight in duct-ligated-sclerosed pancreatic tissue(6). Recently, it has been shown that a highly purified growth hormone preparation administered intravenously to normal men induces a rapidly-occurring, transient hyperglycemia(8) which resembles that caused by hyperglycemic factor given intravenously to several species including man(9). Bornstein *et al.*(10) reported that pancreatico-duodenal vein blood (but not femoral vein blood) from cats made diabetic with growth hormone contains a hyperglycemic-glycogenolytic substance when tested in the alloxan diabetic-hypophysectomized-adrenalectomized rat. These experiments are consistent with the hypothesis that pituitary growth hormone stimulates the release of hyperglycemic factor from the islets of Langerhans as suggested by Bornstein *et al.*(10). The fact that growth hormone may elicit its characteristic effects in depancreatized animals(11) is not conclusive evidence against this hypothesis, since hyperglycemic factor has been isolated from the gastric and duodenal mucosa of the dog and rabbit(6).

TABLE I. Assays of Hyperglycemic Factor (HGF) Preparations for Glycogenolytic Activity in Pigeon Liver Slices Incubated in 2.1 ml Saline-Phosphate Buffer (Ca^{++} Free).

Preparation	Min amt of preparation /ml incubation medium giving max glycogenolytic response, μg
HGF #3	2.4
#5	1.2
#208-65B-257	.7
Insulin used as source of HGF #3 and HGF #5	24

This paper reports briefly on some experiments which were done to determine whether the growth stimulating action of pituitary growth hormone could be mediated through hyperglycemic factor.

Experimental. The purified hyperglycemic factor preparations used in Exp. A and B were prepared from a sample of a low ash insulin† by the method of Sutherland *et al.*(12). The hypoglycemic action of insulin is completely destroyed during this procedure(13). Fraction No. 3 (HGF No. 3) of the Sutherland method was used in Exp. A and fraction No. 5 (HGF No. 5) was used in Exp. B. The hyperglycemic factor preparation (HGF No. 208-65B-257) used in Exp. C, prepared by Dr. Alfred Staub of the Lilly Research Laboratories, contained less than 0.1 unit of insulin per mg. All 3 hyperglycemic factor preparations were assayed by an *in vitro* method modified from that of Sutherland *et al.*(13). The modifications were the use of pigeon liver instead of rabbit liver and an in-

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† Kindly supplied by Dr. R. G. Romans of the Connaught Medical Research Laboratories, Toronto.

‡ Obtained from the Hormone Assay Laboratory, Chicago, Ill.

TABLE II. Exp. A: Effect of Hyperglycemic Factor #3, and Various Growth Hormone Preparations on the Proximal Tibial Epiphyseal Cartilage of Hypophysectomized Rat. (4 daily inj.)

Group	No. of animals	Daily dose in μg	Cartilage plate response, μ ± S.E.*	Significance of response: P†
Control	5	0	109 ± 11.6	
Horner growth hormone #20-1-2-J51	5	10	150 ± 11.8	<.05
Li growth hormone	5	10	175 ± 17.5	<.02
Hyperglycemic factor #3	4	25	144 ± 10.6	<.1
" " "	2	100	176, 178‡	

* The low values in this experiment were due to a different method of making measurements from that used in Exp. B and C.

† Obtained from Fisher's table of t.

‡ Individual values.

TABLE III. Exp. B: Effect of Hyperglycemic Factor #5, and Various Growth Hormone Preparations on the Proximal Tibial Epiphyseal Cartilage of Hypophysectomized Rat. (4 daily inj.)

Group	No. of animals	Daily dose in μg	Cartilage plate response, μ ± S.E.	Significance of response: P*
Control	10	0	166.9 ± 6	
Li growth hormone	11	10	253.6 ± 7	<.001
Horner growth hormone #20-1-2-J51	11	10	246.8 ± 4.7	<.001
Hyperglycemic factor #5	9	10	202.5 ± 7.1	<.01
" " "	9	40	201.1 ± 4.8	<.001

* Obtained from Fisher's table of t.

cubation medium of 2.1 ml of a saline-phosphate buffer(14) (Ca^{++}-free) instead of 1.2 ml of chloride-phosphate buffer. Glucose determinations in the assays were done by the Hagedorn-Jensen method(15). Female rats[‡] of the Sprague-Dawley strain hypophysectomized at 26-28 days of age were used. They were fed on a diet of canned dog food, whole wheat bread, milk, and 5% glucose. Injections of hyperglycemic factor, growth hormone or saline (controls) were begun 12-14 days after hypophysectomy. They were given intraperitoneally in a volume of 0.5 ml once daily for 4 days (Exp. A and B) or for 9 days (Exp. C). Twenty-four hours after the last injection the animals were killed with chloroform and the tibias examined for changes in the width at the proximal epiphyseal cartilage according to the method of Evans *et al.* for the bioassay of hypophyseal growth hormone(17).

Four highly purified growth hormone preparations[§] were tested for activity along with the hyperglycemic factor preparations: 1) Horner Growth Hormone No. 20-1-2-J51: prepared from beef pituitaries by the Wilhelmi method(18). 2) Li Growth Hormone: the electrophoretically homogeneous preparation of Dr. C. H. Li. 3) Horner Growth Hormone No. PR-1: prepared from hog pituitaries by the Rabens method(19). 4) Armour Growth Hormone No. 264-159A.

Results. Table I shows the results of the assays for glycogenolytic activity of the purified hyperglycemic factor preparations and of the insulin used as the source of HGF No. 3 and HGF No. 5. HGF No. 3 was 10 times and HGF No. 5 20 times as active as the source material. HGF No. 208-65B-257 was the most active of the three.

Tables II, III and IV summarize the results of the tibia test growth assay. Two of the 3 hyperglycemic factor preparations in daily doses of 10 μg, and 40 μg caused statistically significant increases (31-36 μ) in the width at the proximal epiphyseal cartilage of the tibia as compared with controls. HGF

[§] Kindly donated by Dr. L. Mitchell of Frank W. Horner, Ltd., Montreal.

TABLE IV. Exp. C: Effect of Hyperglycemic Factor #208-65B-257, and Various Growth Hormone Preparations on Proximal Tibial Epiphyseal Cartilage of Hypophysectomized Rat. (9 daily inj.)

Group	No. of animals	Daily dose in μg	Cartilage plate response, $\mu \pm$ S.E.	Significance of response: P*
Control	8	0	137.8 ± 2.9	
Horner growth hormone #20-1-2-J51	8	10	185 ± 3.5	<.001
Horner growth hormone #PR-1	8	10	173 ± 1.5	<.001
Armour growth hormone #264-159A	8	10	162 ± 2.7	<.001
Hyperglycemic factor #208-65B-257	7	10	169 ± 7.9	<.01
Horner growth hormone #20-1-2-J51	8	25	256 ± 7.3	<.001
Horner growth hormone #PR-1	8	25	190 ± 2.3	<.001
Armour growth hormone #264-159A	8	25	184 ± 2.7	<.001
Hyperglycemic factor #208-65B-257	5	40	150 ± 4.9	<.05

* Obtained from Fisher's table of t.

No. 3 caused substantial increases but owing to the small numbers of animals used statistical significance could not be established. The tibial response was roughly parallel to the glycogenolytic potency of the 3 hyperglycemic factor preparations, *i.e.*, the order of potency of the preparations was the same by the 2 assay methods. In 2 of the 3 experiments (B and C) increasing the dose of hyperglycemic factor did not increase the tibial response. In one experiment (A) there was a substantial increase in the tibial response when the dose of hyperglycemic factor was raised but in this group only 2 of the 5 animals survived the injection period. In all experiments doses of hyperglycemic factor greater than 25 μg proved toxic (mortality 20-60%).¶ The responses obtained with the Li Growth Hormone (Tables II and III) were somewhat less (by about 25%) than that reported in the literature(17).

Discussion. It appears that small amounts of purified pancreatic hyperglycemic factor are capable of increasing the width of the epiphyseal cartilage of the hypophysectomized rat, an effect which is characteristically obtained with pituitary growth hormone(17). However, in only one of the 3 experiments was an increase in response observed when the dose of hyperglycemic factor was raised. This may have been due to the toxicity of the preparations in doses greater than 25 μg. It may also be that hyperglycemic factor belongs to a group of hormones (thyroxin, prolactin, and testosterone) which at certain dose levels increase the width of the cartilage plate 20-30 μ above control levels but not more when the dose is increased(17). The possibility remains that both the diabetogenic and growth-stimulating actions of growth hormone are mediated, in part or wholly, through the release of hyperglycemic factor from the pancreas and gastrointestinal tract.

The observation that hyperglycemic factor increases the width of the epiphyseal cartilage is in itself of some theoretical interest. Sutherland and Cori(20) have demonstrated that hyperglycemic factor causes a rapid increase in the concentration of phosphorylase in liver tissue. Significant amounts of phosphorylase as well as other enzymes of phosphorylative glycogenolysis have been found in preosseous, epiphyseal cartilage(21,22), and phosphorylative glycogenolysis appears to be essential for the *in vitro* calcification of cartilage(21). Cartilage cells preparatory to calcification accumulate large cytoplasmic stores of glycogen which disappear just prior to or coincident with the appearance of bone mineral in the adjacent cartilage matrix (23,24). If hyperglycemic factor stimulates glycolysis by causing an increase in the phosphorylase activity of tissues it might be that this is the mechanism whereby it stimulates growth. It is of interest, in this connection, that there is a disappearance of glycogen during cell growth and mitosis(25) and that increased rates of glycolysis have long been associated with the accelerated growth rates characteristic of tumor and embryonic tissue as compared with normal adult tissue.

¶ The animals often went into coma following the injections. Some could be revived by glucose administered intravenously. In human experiments, done since this paper was submitted, highly purified preparations of hyperglycemic factor (containing less than 0.005 unit of insulin/mg) caused hypoglycemia, following the usual hyperglycemia. Recent work by Foa(16) suggests that hyperglycemic factor induces a release of insulin from the pancreas.

Summary. Purified preparations of pancreatic hyperglycemic factor have been shown to increase the width of the epiphyseal cartilage of the hypophysectomized rat when assayed by the tibia test used for the bioassay of pituitary growth hormone. The activity of the preparations by this assay parallels their glycogenolytic activity *in vitro*. These observations are discussed from the standpoint of the mode of action of pituitary growth hormone and the process of growth at the enzyme level.

Special acknowledgment is due Professor J. H. Quastel, Director, The Research Institute, Montreal General Hospital, for his interest in this work, and for providing laboratory facilities for these experiments, and to Dr. L. Mitchell, Director of Research, Frank W. Horner Ltd., Montreal. Thanks are due Cameron Bruce for the tibia test assays and Rosalind Fridhandler for the glucose determinations.

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Caries Potentiating Effect of Ethylene Diamine Tetraacetic Acid in the Rat.*
(20030)

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Because of its strong metal chelating properties, ethylene diamine tetraacetic acid (EDTA) has received wide application in the study of biochemical reactions requiring metal salts for continued normal activity. Hence, the compound has proved valuable in the study of various calcification and decalcification processes. Studies *in vivo* of decalcifying agents on rat molar tooth surfaces have shown that dilute EDTA solutions adjusted to pH 6.5 have been the most active tried(1).

In the classical studies of W. D. Miller(2) which still serve as a working hypothesis for basic research in the etiology of dental caries, decalcification of dentin and enamel is accorded a prominent role. It was the purpose of this study to investigate the caries potentiating effect of a strong decalcifying agent by adding EDTA to a cariogenic rat diet.†

Experimental and results. Weanling rats of the Holtzman strain were used throughout and the diet and drinking fluids were administered *ad libitum*. At the end of 100 days the rats were sacrificed, the heads were autoclaved, and the molar teeth were charted for caries according to Cox, Dodds, Dixon, and Matuschak(4).

Preliminary study of effect of EDTA in diet and drinking fluid. Eighty rats representing 20 litters were distributed by litter into 2 groups of 40 rats each and housed 10 rats to a cage. Group I (control) received cariogenic diet 585‡ and distilled water. Group II (experimental) received diet 585 containing 0.5% EDTA (diet 585V) and a 0.1% solution of EDTA calculated as the tetrasodium salt adjusted to pH 6.5 as the drinking fluid. The caries scores are presented in Table I. Comparison of the difference of the means for carious areas and caries score in Groups I and II yielded a "t" value of 3.90 and 3.50 respectively ($p < 0.01$). The production of the normal yellow-orange pigment of the incisors was markedly inhibited so that they appeared ivory in color, but unlike the effect produced by fluoride, showed no striations, pitting, or chipping.§

TABLE I. Caries Score of Rats Receiving EDTA in Diet and Drinking Fluid.

Group	I	II
Diet	585	585V
Drinking fluid	H_2O	EDTA
No. of rats	40	34
Rats with caries %	100	100
Carious teeth per carious rat	4	5.1
" areas " " "	7.8	11.8
Caries score " " "	16.3	24.7

Relation between decalcification potential and caries. In an attempt to evaluate the decalcification action of EDTA in caries potentiation, other *in vivo* decalcifying agents(1) were administered in both the diet and drinking fluid to rats receiving diet 585. Litter mates from 20 litters representing 120 rats were divided into 3 groups of 40 rats per group and housed 10 rats per cage. Group I received diet 585 and distilled water; Group II received diet 585 containing an added 1% so-

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* The EDTA was kindly supplied as Sequestrene AA by the Alrose Chemical Co., Providence, R. I. We are indebted to Dr. Elias Elvove and to Mr. Charles G. Remsburg for mineral analysis of diet 585; to Mr. Arnold R. Stull, Jr., Laboratory of Biochemistry and Nutrition, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, for the hematocrit determinations.

† Recently it was reported in abstract(3) that a cariogenic diet containing 0.2% ethylene diamine tetraacetic acid increased the incidence and severity of smooth surface caries in rats.

‡ Formulated by R. M. Stephan of this Institute, and described in(5). Mineral analysis gave the following concentrations based on fresh weight of diet: Ca, .29; Mg, .06; Fe, .006; Mn, .010% respectively.

§ Macroscopic evidence of striations is seen at approximately 10 ppm F, whereas 100-200 ppm F are necessary to produce pitting and chipping.

TABLE II. Effect of Addition of Iron to a Cariogenic Diet Containing EDTA.

Group	I	II	III	IV
Diet	585	585V	585V+Fe	585+Fe
Drinking fluid	H_2O	H_2O	H_2O	H_2O
No. of rats	19	14	20	18
Rats with caries %	100	100	100	100
Carious teeth per carious rat	4.1	4.8	4.6	4.1
" areas " " "	5.4	9.9	7.4	5.6
Caries score " " "	11.6	22.1	15.1	10.7

dium citrate dihydrate (diet 585C) and a solution of 0.25 N sodium citrate dihydrate adjusted to pH 6.5 as the drinking fluid. Group III received diet 585 containing an added 1% of Calgon[‖] (diet 585CG) and a solution of 2% Calgon adjusted to pH 6.5 as the drinking fluid. Groups II and III showed only a slight increase in the carious areas and caries score per carious rat. These differences, however, are not significant statistically ($p > 0.05$). No interference with normal incisor pigment could be seen in any of the groups. It appears, therefore, that citrate and calgon did not significantly potentiate caries on diet 585, although extensive decalcification of the molar tooth surfaces was apparent.

Reversal of effects of EDTA by addition of iron. It has been reported that the incisor pigment is due in large measure to its iron content which may be reduced by as much as 50% in anemia(6). Since the inhibition of incisor pigmentation indicated iron chelation by EDTA, an attempt was made to counteract the effects of EDTA by the addition of iron as the iron and ammonium citrate salt (Mallinckrodt, USP XI, brown scales). Ninety-six rats representing 25 litters were distributed according to litter into 4 groups of 24 rats each, and housed 2 rats per cage. Group I received diet 585. Group II received diet 585V. Group III received diet 585V containing an added 0.6% iron and ammonium citrate, and Group IV received diet 585 supplemented with the same amount of iron salt used by Group III. All rats received distilled water. Hematocrit values were determined on representative rats of each group. The caries data are presented in Table II.

Both the number of carious areas and the caries score per carious rat showed nearly a 2-fold increase when diet 585 contained an added 0.5% EDTA. The addition of iron to this diet (585V) produced a slight but not significant reversal of the effect of EDTA as indicated by the somewhat lowered caries score in Group III as compared with Group II. Adding 0.6% iron and ammonium citrate as a source of Fe to diet 585, had no appreciable effect on either the incidence or severity of caries. It has also been previously reported by McClure(7) that iron appears to have no pronounced effect on induced rat caries, when added to a cariogenic diet.

Supplementation of diet 585V with 0.6% iron and ammonium citrate as a source of iron seemed to completely prevent the hematocrit lowering and the incisor pigmentation inhibiting effect of EDTA. The mean hematocrit values found for Groups I-IV were 43.0, 29.7, 43.2, and 43.0, respectively.

To corroborate the effect of EDTA when added to the diet alone an additional 48 rats representing 24 litters were divided into 2 groups of 24 rats per group and housed 2 per cage. Group I received diet 585 and Group II received diet 585V with distilled water available *ad libitum* to both groups. The mean hematocrit values on representative rats of Groups I and II of 43.1 and 35.1 respectively were significantly different at the 1% level ($p < 0.01$). The carious areas per carious rat were 2.9 and 5.7 respectively, and the caries scores per carious rat were 5.1 and 12.7 respectively. When a 0.1% EDTA solution adjusted to pH 6.5 was administered as the drinking fluid to 24 rats receiving diet 585, no significant caries potentiating effect was apparent when compared to litter mates receiving diet 585 and distilled H_2O. In ad-

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[‖] Manufactured by Calgon, Inc. Pittsburgh, Pa., and is presumed to be a hexametaphosphate.

TABLE III. Composite Data on the Caries Potentiating Effect of EDTA.

	10 rats/cage		2 rats/cage	
Diet	585	585V	585	585V
No. of rats	78	34	41	32
Rats with caries %	97.4	100	87.8	100
Carious teeth per carious rat	3.5	5.1	3.5	4
" areas " " "	5.9	11.8	4.2	7.5
Caries score " " "	11.8	24.7	8.5	16.8

dition, no interference with normal incisor pigmentation or lowering of the hematocrit value was observed.

The combined studies reported thus far comprise 344 rats. For composite comparisons of the effect of EDTA added to a cariogenic diet the data may be placed in 2 groups, *i.e.*, with rats housed 10 to a cage and with rats housed 2 to a cage. The data are presented in Table III. This separation of data is made since no reports have appeared on the effect of crowding on experimental rat caries.

It appears, therefore, that the carious areas and caries score are increased approximately 2-fold when EDTA is present in the diet. The difference in the means is statistically significant at the 1% level, indicating a marked caries potentiating effect of EDTA when added to cariogenic diet 585.

Discussion. No adequate explanation at present accounts for the apparent caries potentiating effect of EDTA in the rat. A lowered hematocrit value is produced as well as a marked inhibition of incisor pigment formation, and both these effects appear to be reversed by the addition of iron to the diet. The caries potentiating effect of 0.5% added EDTA to the diet does not seem to be associated with its decalcifying ability since other known decalcifying agents when added to the cariogenic diet 585 do not significantly increase the severity of caries. It has been suggested by the work of McClure and Ruzicka (8) that the non-acid decalcifying effect of the citrate anion may possibly play a role in the etiology of dental caries. In the present study, no significant increase in the severity of caries appears to be occasioned by the addition of citrate to the diet and drinking fluid. In addition preliminary studies have indicated that no decalcification, similar to that reported in previous studies(9), is found when EDTA is added to the diet alone.

Since both fluoride and iodoacetate inhibit rat caries(10), enzymatic reactions have been implicated in caries etiology. It appears possible that EDTA may have an effect on such enzyme systems linked to the carious process and requiring trace metals. According to our results one of these elements does not seem to be iron. The addition of 0.5% EDTA to cariogenic diet 585 has produced some diarrhea and a depression of weight so that the possibility must also be considered that EDTA *per se* may exert some secondary or indirect caries potentiating effect due to its toxicity. The effects of EDTA described appear to be associated with its presence in the diet, although its effect in the drinking fluid cannot be entirely precluded at this time.

It appears, therefore, that EDTA may be a valuable tool in investigating the possible role of mineral deficiency and cation antagonism in the etiology of experimental dental caries.

Summary. The addition of 0.5% EDTA to a cariogenic diet appeared to potentiate caries to a marked degree in the white rat. Concomitantly, the hematocrit value is significantly lowered, and almost complete inhibition of incisor pigmentation is found. These effects of EDTA do not seem to be associated with its ability to decalcify rat molar teeth *in vivo*. Citrate and calgon added to a cariogenic diet and present in the drinking fluid do not significantly increase the severity of caries.

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Some Observations of Effects of Intravenous Fat Emulsions on Erythrocyte Fragility. (20031)

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After the administration of intravenous fat emulsions, a moderate but self-limited anemia has frequently been reported. Mann *et al.* (1,2) noted the occurrence of moderate anemia in dogs which were given corn and coconut oil emulsions. Anemia also occurred in rats given both coconut and corn oil emulsion, and in 2 human subjects who received coconut oil emulsion. In the latter report, increased osmotic fragility of the red cells was noted. The authors found that the phosphatide used as stabilizer could alone produce changes of the same magnitude. Meng and Freeman(3) observed a slight anemia in a group of dogs given stabilizing agents, but in only one of 5 dogs given complete emulsion. Meng(4) observed no anemia in infused rabbits. Van Itallie *et al.*(5) stated that preliminary studies of red cell fragility failed to show changes in the integrity of the red cell membrane, but no details were given. Lerner, Chaikoff, and Entenman(6) likewise reported the occurrence of anemia in a group of dogs given intravenous fat for prolonged periods as did Dunham and Brunschwig(7) and Collins (8). The latter believed that it was probably not hemolytic since reticulocytosis did not occur. In this laboratory, significant decreases in hemoglobin, red blood cell count and hematocrit were observed in a group of normal dogs after one to 2 weeks of daily intravenous infusion of fat(9). In contrast, Shafiroff *et al.*(10,11) found no hematologic changes after single infusions of fat emulsion in humans and stated that in no case did infusion result in a positive test for urine urobilinogen.

The mechanism of the observed anemia remains unexplained. It could be inferred from the nature of the infused substances that this is a hemolytic anemia. Surface active stabilizers are used in many of the emulsions and these materials might well alter the red cell membrane. The emulsified fat itself might conceivably alter the interfacial relationships between the erythrocyte and its environment. In addition, minute amounts of other surface active agents such as free fatty acids may be present in the emulsion or be liberated after infusion. Some of the hematologic effects of intravenous fat emulsions given to dog and man and the possible significance of these effects are reported here.

Procedure. The fat emulsions used were from 2 sources: Emulsion I is a 10% emulsion of synthetic fats stabilized with Span 20, sodium cholate, soybean phosphatides and Demal—14.* Emulsion II is a 15% olive oil emulsion stabilized with 0.6% soybean phosphatide and 1.0% Demal—14†. The subjects in these experiments were 2 burn patients, one convalescent and the other in the final stages of skin grafting. The dog used was a normal male weighing 22 kg. All subjects were studied in the fasting state. The patients received 500 ml of emulsion and the dogs 300 ml at a rate of approximately 4 ml per minute.

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* Generously supplied by Dr. H. C. Meng.

† Generously supplied by Dr. F. J. Stare.

Heparin was used as an anticoagulant. Blood samples were drawn before infusion, immediately at the end of the infusion and at appropriate intervals thereafter. All blood was drawn with maximum precaution to prevent artificial hemolysis. Venapuncture was accomplished with a 15 gauge needle and after flow of blood was established the initial sample was discarded and the sample for analysis was drawn into a mineral oil coated syringe and delivered into a similarly oiled test tube. The specimens were immediately centrifuged and the plasma separated. Twenty-four hour stool specimens were collected for urobilinogen determination. Separation of the daily samples was accomplished by the use of carmine markers.

Methods. 1. Plasma hemoglobin was determined by a benzidine procedure to be reported elsewhere(12). 2. The mechanical fragility of erythrocytes in whole blood was quantitated as follows: 4 ml of heparinized whole blood was pipetted into each of two 13 by 100 mm glass stoppered test tubes containing 2 glass beads. The tubes were shaken for 15 and 30 minutes in a Fisher Clinical Shaker at a rate of 275 oscillations per minute after which they were centrifuged. The hemoglobin concentration of the supernatant plasma was then determined. The 15 and 30 minute values in mg % are averaged and the average is referred to as the fragility index. 3. Plasma "total lipid" was measured by a macromodification of the turbidimetric technic of Geyer *et al.*(13). Using the preinfusion blood as a blank, this method measures the increment in total lipid resulting from the infusion. 4. Urobilinogen: 24-hour excretion of fecal urobilinogen was measured by the technic of Schwartz, Sborov, and Watson(14).

TABLE I. Dog Experiments.

Exp.	Min. after infusion	Plasma total lipid, mg %	Plasma Hgb, mg %	Fragility index
A	P*	0	1.6	218
	0	3647	18.8	5050
	130	1703	14.2	5835
	305	368	3.9	3550
B	P*	0	0	54
	0	575	6.5	5690
	120	75	4.1	1094
	240	11	0	60

* P = Preinfusion.

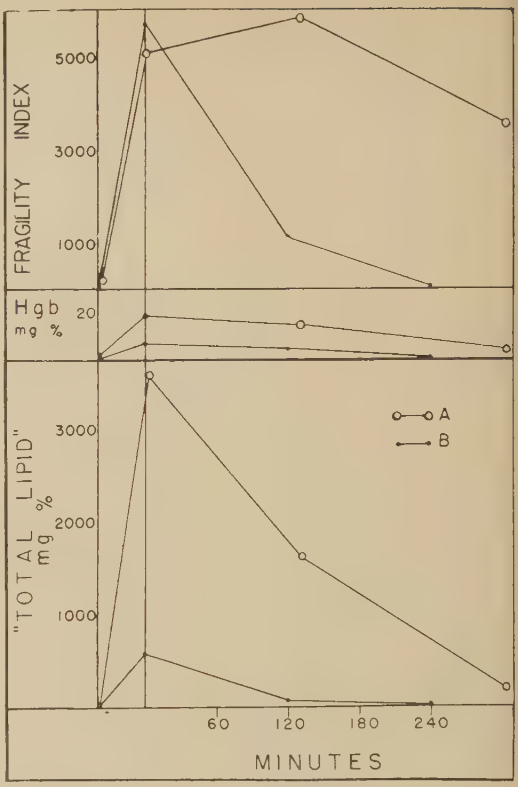

FIG. 1. Dog experiments.
Emulsion II used in Exp. A (open circles).
" I " " " B (closed ").

Results. Dog experiments. Two dog experiments are reported (Table I, Fig. 1). In Exp. A, emulsion II was used and in Exp. B emulsion I was infused. In one case (Exp. A) the fat emulsion was inadvertently given at a very rapid rate which resulted in the extremely high plasma "total lipid" level of 3700 mg %.

There was an immediate, very marked rise in the fragility index to above 5,000 in both experiments. The average control fragility index determined on 18 samples of blood from the same dog given glucose infusions in place of fat was 72.6 with a range of 10 to 276. The *fragility index* gradually returned toward normal as the plasma "total lipid" decreased, but was still considerably elevated as long as visible lipemia was present ("total lipid" above 50 mg %). In both experiments a small degree of spontaneous hemolysis oc-

TABLE II. Human Experiments.

Exp.	Min. after infusion	Plasma total lipid, mg %	Plasma Hgb, mg %	Fragility index
A	P*	0	0	31
	0	233	49.5	1870
	60	96	6.2	2140
	120	17	5.1	108
	240	0	8.9	87
B	P*	0	0	27
	0	913	23.4	14500
	60		9.6	12700
	120	484	3.1	10000
	240	352	6.8	5500
C	P*	0	0	37
	0	478	1.7	2625
	60	396	1.7	1925
	120	230	10.9	268
	180	90	.5	297

* P = Preinfusion.

curred. Maximum hemoglobin values of 19 mg % and 6.5 mg % were found in the plasma. Plasma hemoglobin fell with clearing of the fat from the plasma. There was no change in daily fecal excretion of urobilinogen in either experiment.

Human experiments. Three experiments were carried out on 2 patients. The results are plotted in Table II, Fig. 2. In A and B fat emulsion II was used and in Exp. C emulsion I was infused. The fragility rose to 14,500 in one experiment indicating hemolysis of almost the entire red cell mass during shaking. The average of 5 control indices was 58 with a range of 27 to 108. Only minimal spontaneous hemolysis occurred in Exp. C, but in A a plasma hemoglobin level of 50 mg % was reached. In this experiment there was increase in body temperature to 100° associated with symptoms of nausea and apprehension. In neither of the other experiments did fever occur although in Exp. B a plasma hemoglobin level of 22 mg % was reached.

Table III illustrates the 24-hour fecal excretion of urobilinogen. In Exp. A there was a rise in urobilinogen excretion. In Exp. B urobilinogen excretion also increased to a lesser degree, proportionate to the observed differences in spontaneous hemolysis. The change in urobilinogen excretion in Exp. C is not significant.

Discussion. Decreased *in vitro* resistance of the erythrocyte to mechanical stress after infusion of fat emulsions has been demonstrated. In earlier experiments in this laboratory in which intravenous fat was given to

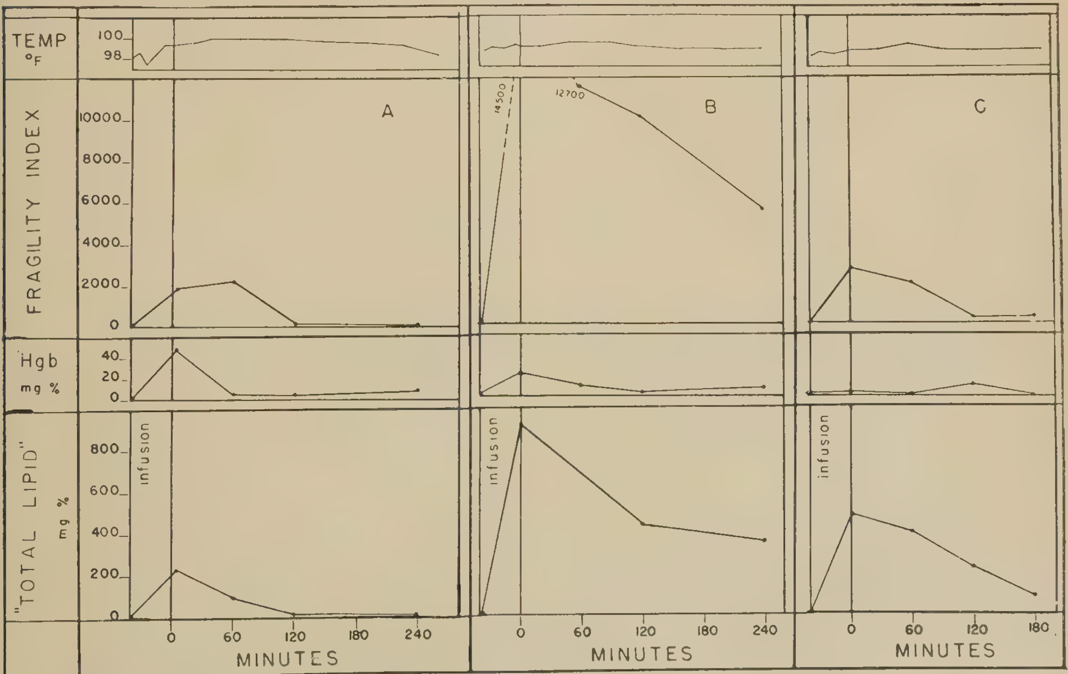

FIG. 2. Human experiments. Emulsion II used in Exp. A and B. Emulsion I used in Exp. C.

TABLE III. Daily Urobilinogen Excretion in Three Human Subjects.

Day	Fat infusion	24-hr urobilinogen excretion, mg/day
1	—	152
2	—	132
3	A	224
1	—	85
2	—	84
3	B	128
4	—	77
1	—	30
2	—	30
3	C	43
4	—	23

Day of fat infusion is indicated by letter corresponding to exp. in Fig. 2.

dogs, mechanical and osmotic fragility was determined in the classical manner using washed red cells in very dilute suspension. In none of these trials was any change in fragility observed. The decreased resistance of the erythrocyte exists only while the cell is in lipemic plasma. Van Itallie *et al.*(5) give no details of technic when they state that no change in the integrity of the red cell membrane can be shown. It appears quite likely that differences in technic might explain the divergent results.

Since increased fragility can be easily reversed by washing *in vitro* and since the fragility index rapidly falls with *in vivo* clearing of the plasma fat, there is no evidence for an irreversible change in the red cell itself. Although this increased fragility could certainly predispose to intravascular hemolysis, the actual occurrence of such hemolysis would remain an inconstant phenomenon. The inconsistency of the occurrence of anemia in published studies lends support to this hypothesis. Of the experiments reported here, only one showed a great degree of intravascular hemolysis.

In view of the extreme fragility of the cells in lipemic plasma, measurable elevation of plasma hemoglobin may be produced in the handling of the blood specimens. However, in our experiments the parallel increases in fecal urobilinogen excretion in the 2 cases where plasma hemoglobin rose above 10 mg % rule against this possibility.

Is the observed increased red cell fragility a function of hyperlipemia *per se,* or is it a phenomenon associated only with parenterally administered fat emulsions? Increased bile pigment excretion has been reported to occur as a result of high fat diets(15,16). In addition, it has been demonstrated that lipemic lymph and plasma cause increased erythrocyte fragility and hemolysis(17-19).

The physical state of hyperlipemia itself is common to all of the situations described here and in the literature. The highly emulsified fat might well distribute itself around the red cell and alter membrane relationships in such a way that fragility is increased.

Shafiroff *et al.*(20) have demonstrated the occurrence of intravascular hemolysis and severe reactions after the infusion of relatively soluble short chain fats. These may be easily saponified and contribute surface active products of hydrolysis. Others(21) have correlated the hemolytic action of fatty chyle with its free fatty acid and soap content.

Fever is the most common reaction to intravenous fat administration(1,5,10,22) and has been reported from every laboratory investigating the use of intravenous fat emulsions. Current estimates put the rate of reaction at 15%(23) to 33%(22) of infusions. The reaction is erratic in occurrence and emulsions may cause fever in one individual but not in another. Most diseases associated with acute intravascular hemolysis are characterized by the occurrence of fever. It also appears reasonable to infer that the febrile response to intravenous fat is a reaction to intravascular hemolysis. In the report of Shafiroff *et al.* (20) the intravascular hemolysis observed was held responsible for the accompanying symptoms, including the temperature response. In our experiments, the only febrile reaction was associated with the greatest degree of hemolysis observed. Further correlations of this type should be sought. If the occurrence of intravascular hemolysis is a chance phenomenon as suggested, it would explain the erratic, unpredictable occurrence of the febrile response to intravenous fat emulsions.

Summary. 1. Intravenous infusion of fat emulsions causes increased mechanical fragility of dog and human red blood cells. 2. Spontaneous intravascular hemolysis has been

seen in humans who have received these emulsions. 3. The possible causes of this phenomenon and its possible relationship to the "pyrogenic" effects of intravenous fat are discussed.

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Production of Group A Streptococcal Cervical Lymphadenitis in Mice.[*][†]

(20032)

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(Introduced by C. G. Harford.)
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The fact that hemolytic streptococcal infections are implicated in the causation of rheumatic fever is well documented(1-3). Further, there is much evidence to suggest that rheumatic fever occurs as a result of an inappropriate immune response on the part of the host to the streptococcus or its products(4,5). Nevertheless, the pathogenesis of rheumatic fever remains obscure, its elucidation being seriously handicapped by the lack of a suitable experimental analog. The development, therefore, of an affection in experimental animals, bearing sufficient resemblance to rheumatic fever in man to satisfy the most stringent criteria, might well constitute a significant advance toward definition of the mechanism by which the human disease arises. One avenue of attack on this problem is represented by the attempt to simulate in experimental animals a sequence of events comparable to that which is assigned importance in human rheumatic fever; namely, repeated infections of the upper respiratory tract with group A streptococci(6). In order to pursue this par-

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[†] Presented at Meeting of the Commission on Streptococcal Diseases, Cleveland, April 2, 1952.

[‡] Life Insurance Medical Research Fund Fellow when this work was done.

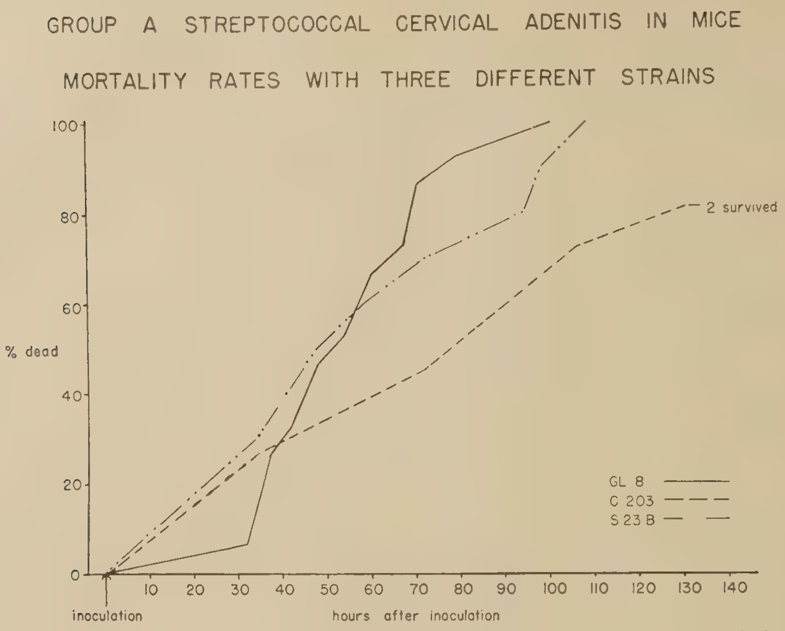

FIG. 1. Mortality curves for mice with cervical lymphadenitis. Ten animals were infected with S23B, eleven with C203 and fifteen with GL-8 (IGL-23).

ticular investigative approach, it is essential to develop a method by which group A streptococcal infection can be achieved via the upper respiratory route in an experimental animal.

The present paper describes the successful production of group A cervical lymphadenitis in mice by means of a modification of the intranasal technic described by Sonkin(7). In addition, the pathogenesis of the infection is delineated on the basis of systematic histologic observations.

Methods. Swiss mice of the Webster strain, weighing between 15 and 20 g, were injected intraperitoneally with 0.3 ml of a 2.5% solution of chloral hydrate, and then allowed to inhale ether until deeply anesthetized. Group A streptococci (strains C203, GL-8(IGL-23) or S23B)[§] were grown in beef infusion broth containing 10% sheep serum. A 4-hour culture was centrifuged in the cold at 3000 RPM for 30 minutes, the supernate removed, and the organisms resuspended in gelatin-Locke solution. Intranasal inoculation was performed by introducing in turn, into each nostril, a blunt, smooth 27 gauge needle attached to a 0.25 ml syringe and delivering 0.01 ml of bacterial suspension.[‖] The mice were allowed to recover from the anesthesia and were kept in groups of 5 or 6 in suitable cages. Several experiments were performed in which animals were inoculated with a given strain of streptococcus and observed until death, so that mortality curves could be computed. Other experiments were done in which mice were killed at given intervals, in order that systematic gross and microscopic observations could be made. In these pathogenesis studies three or more mice were sacrificed at the times indicated. The affected nodes were removed immediately, fixed in Zenker-formol solution, sectioned and stained by the Giemsa technic. Heart's blood was used for culture and streaked on blood agar poured plates.

Mortality. With the technics employed the infection induced was almost always fatal

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[§] All 3 strains were received from investigators at the Hospital of the Rockefeller Institute as follows: C203 from Dr. Rebecca Lancefield in 1943, GL-8(IGL-23) from Dr. Maclyn McCarty in 1947, and S23B from Dr. Sidney Rothbard in 1948.

[‖] Tenfold dilutions of the resuspended culture were made, and blood agar plates were poured with 1.0 ml aliquots of the 10^{-6}, 10^{-7}, 10^{-8} dilutions. The inoculum was calculated to contain approximately 5×10^6 chains.

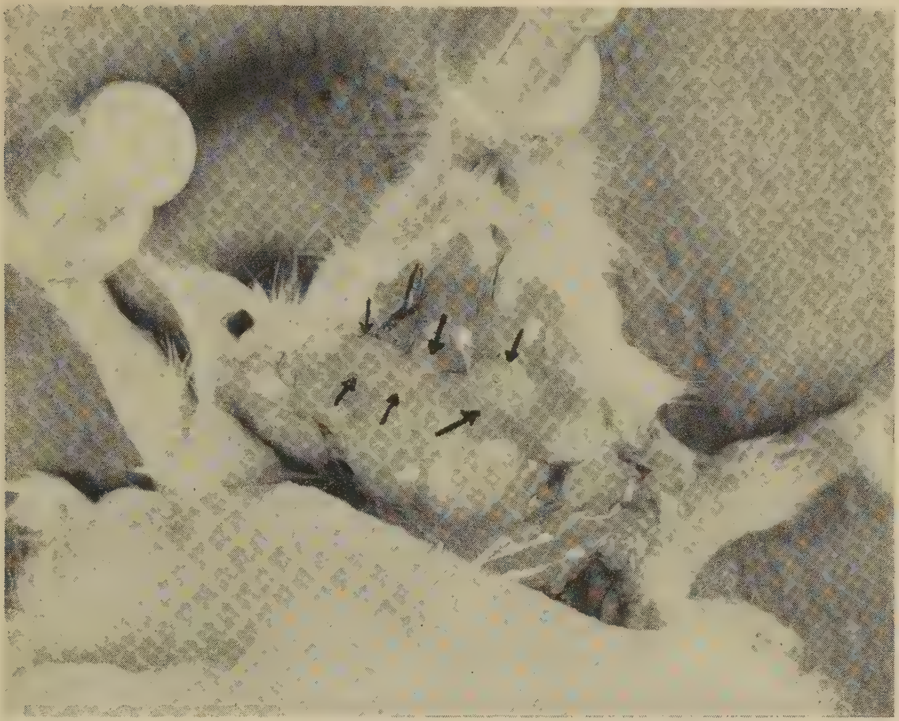


FIG. 2. Gross appearance of involved cervical lymph nodes (arrows). Animals sacrificed forty-eight hr after intranasal inoculation.

within 120 hours. In Fig. 1 composite data from three mortality experiments, employing 10, 11, and 15 mice respectively, are shown.

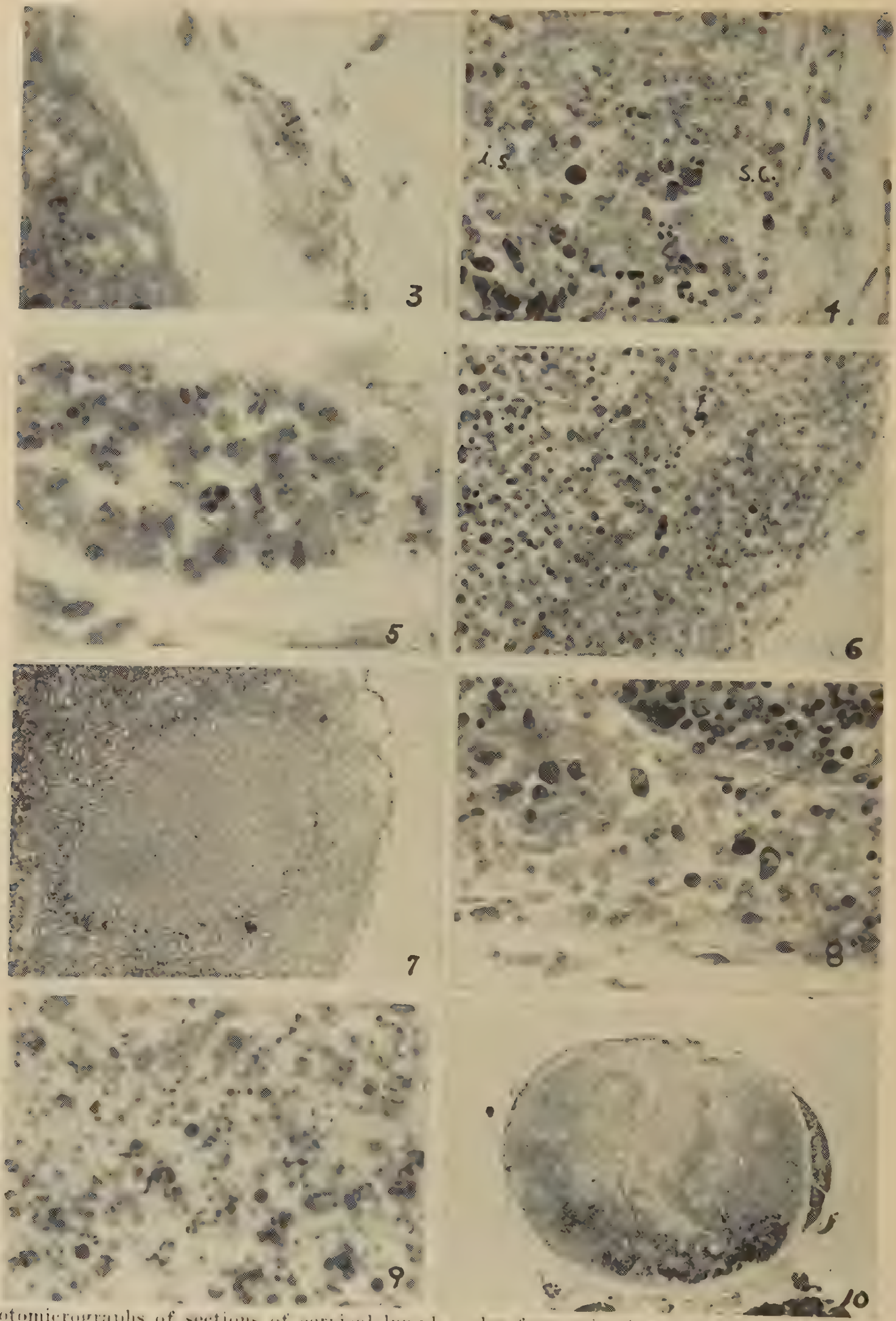
Gross observations. In almost all instances the superficial cervical lymph nodes were primarily affected, often symmetrically. In 12 to 20 hours after inoculation the nodes were enlarged, although maximum increase in size usually occurred between 24 and 48 hours. The degree of enlargement was variable, but often the infected nodes were 2 to 4 times normal size (Fig. 2). Swelling of the nodes was visible, and they could be palpated easily through the intact skin. The nodes of animals dying of the infection were frequently friable, and in some instances frank abscess formation was apparent. Extension of the inflammatory process to the contiguous tissues or to the lungs did not occur.

Microscopic observations. Some variation was encountered in the histologic appearance of nodes taken from animals sacrificed at the same interval after inoculation. These observations, therefore, constitute a summary of the usual pattern of changes found at a given time.

No alteration was found in the nodes 6 hours after inoculation, although streptococci were found within lymphatic vessels in the capsule (Fig. 3). These channels presumably represented the afferent pathway by which the organisms reached the node. Within 12 hours after inoculation bacteria had reached the subcapsular sinus, and were entering the intermediary sinuses. A polymorphonuclear exudate was present, and there was active phagocytosis of streptococci by the leucocytes (Fig. 4).

Twenty hours after inoculation, the organisms in the subcapsular sinus were often completely phagocyted by the neutrophilic leucocytes (Fig. 5). The bacteria had advanced farther into the substance of the node, where a leucocytic response was provoked (Fig. 6).

Streptococcal invasion and polymorphonuclear leucocytic exudation often involved much of the node within 30 hours, and markedly altered its architectural pattern. In some



Photomicrographs of sections of cervical lymph nodes from mice inoculated intranasally with Group A streptococci. The sections were stained by the Giemsa technic.

FIG. 3. Streptococci in a capsular lymphatic of a cervical lymph node six hr after intranasal inoculation. × 667.

FIG. 4. Bacteria and polymorphonuclear leucocytes in the subcapsular (s.c.) and intermediary (i.s.) sinuses 12 hr after induction of infection. Phagocytosis of streptococci by leucocytes is already apparent. × 400.

FIG. 5. Phagocytosis of streptococci in the subcapsular sinus by neutrophilic leucocytes 20 hr after inoculation. × 1133.

FIG. 6. Section of the same node shown in Fig. 5. Although the bacteria in the capsule have been phagocyted, other organisms have invaded the substance of the node, and polymorphonuclear leucocytes have appeared in the newly involved areas. × 400.

FIG. 7. Replacement of a portion of a node by an abscess containing neutrophilic leucocytes and bacteria 30 hr after onset of infection. × 67.

FIG. 8. Macrophages in an area in which the active infection has been controlled 48 hr after its initiation. × 400.

FIG. 9. Large numbers of streptococci in another portion of the same node shown in Fig. 8. Here the cellular defenses have lagged. × 1020.

FIG. 10. Low power view of the node from which the sections in Fig. 8 and 9 were taken. Note the destruction of the architectural pattern. × 18.

areas abscess formation was noted (Fig. 7).

Where the infection was controlled, as evidenced by the disappearance of bacteria and neutrophilic leucocytes (Fig. 8), macrophages predominated. Usually such sites were in the periphery where the acute changes first occurred. Frequently within the same node, however, the presence of great numbers of streptococci, outside the phagocytes, indicated that the infectious process was uncontrolled (Fig. 9), a fact further substantiated by the destruction of nodal tissue (Fig. 10). *Bacteriologic findings.* Although in the pathogenesis studies bacteremia was demonstrated to have occurred as early as 12 hours after inoculation, blood stream invasion usually began 18 to 24 hours after induction of infection. Terminally all the animals had positive blood cultures.

Discussion. Laboratory animals are not naturally subject to infections with group A streptococci. Although it has been known for many years that rabbits are susceptible to hemolytic streptococcal skin infections(8,9), it has recently been demonstrated that such infections may be induced by group A strains (10). The production of pneumonic infections in rats with group A streptococci has now also been reported(11). Previous attempts to cause upper respiratory infections in small laboratory animals with group A organisms have been unsatisfactory(12), but this study indicates that cervical lymphadenitis in mice can be induced consistently with group A strains by a modification of the technic previously described by Sonkin(7). Despite the fact that the method employed in the present study is less accurate in regard to the inoculum delivered than in Sonkin's, it is nonetheless adequate for the production of infections of reproducible virulence. The data presented in Fig. 1, compiled from mortality studies with 3 different strains of streptococci, as well as unpublished data for mortality curves for the same strain used in various experiments, support this conclusion.

The inocula employed in this study caused a rapidly progressing fatal infection. In order to study the response to a non-fatal infection, or to evaluate the effects of repeated infections, modification of the inoculum will be necessary unless an effective chemotherapeutic agent, such as penicillin, is to be used.

Intranasal inoculation of mice with streptococci consistently results in grossly detectable lymph node involvement. Symmetrical lymphadenitis, however, is not achieved with uniformity, a fact which limits to some extent the systematic microscopic study of the infection. This situation differs significantly, therefore, from that encountered by Smith and Wood in their study of type I pneumococcal popliteal lymphadenitis in the rat(13), where a single node drained the site of inoculation. Nonetheless, the pathogenesis of group A streptococcal lymphadenitis in mice and of type I pneumococcal lymphadenitis in rats is similar in the following respects: 1) earliest involvement occurs in the subcapsular sinus; 2) polymorphonuclear leucocytes appear promptly in the involved areas and persist in the portions of the node in which bacteria are present; 3) phagocytosis of bacteria by neutrophilic leucocytes¶ becomes apparent as soon as the polymorphonuclear cells appear in the infected areas; 4) as the inflammatory process subsides, macrophages, which herald the onset of the repair process, become the predominant cell in the involved areas; and 5) fibrin formation does not occur.

¶ Because this phenomenon occurs within 12 hours after induction of the infection—a period too brief to allow for the appearance of circulating antibody (14)—it is considered to represent another example of the important host defense mechanism, surface phagocytosis(15).

In contrast to Smith and Wood's observations with type I pneumococci, the following features were noted in this study: 1) there is destruction of nodal architecture by the infectious process with formation of frank abscesses;** and 2) streptococci persist in the involved nodes for many hours. It should be pointed out that in these regards type III pneumococcal infections bear more similarity to group A streptococcal infections than do those produced by type I pneumococci (11,17).

That the infection was confined initially to the lymph nodes was evidenced by the fact that bacteremia usually did not occur until 18 or more hours after the animals were inoculated. Terminally blood stream invasion was encountered in all instances. Examination of contiguous structures and of the lungs failed to show streptococci outside the involved nodes, even in the most fulminating infections. This finding contrasts with that report by Sonkin when group C streptococci were used. With the latter organism, under the conditions employed, cellulitis of the areas surrounding the involved nodes was common.

Summary and conclusions. The study described herewith affords a demonstration of the fact that a small laboratory animal can be infected via the upper respiratory tract with group A streptococci by a simple reproducible method. The lymphadenitis resulting from group A streptococcal infection bears significant resemblance in its histologic pattern to that produced by pneumococci, particularly by type III strains.

** The necrotizing property of group A streptococci(16), a property not common to pneumococcus, probably explains this particular difference.

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Adrenal Weight and Ascorbic Acid Concentration in Alloxan-Injected Rats.

(20033)

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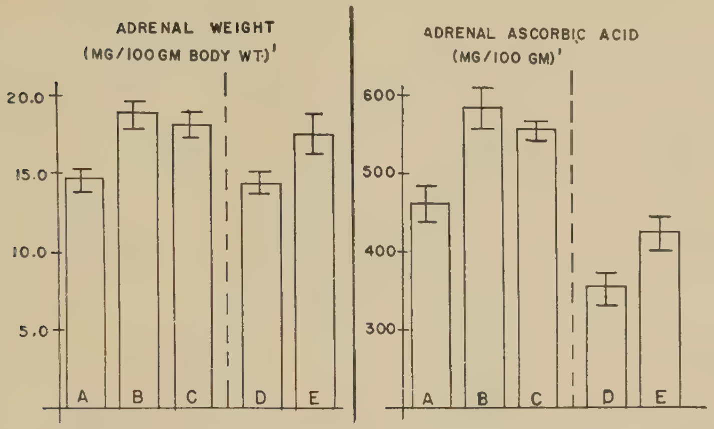
A reduction in adrenal ascorbic acid concentration and/or an increase in wet or dry weight of the adrenal glands are commonly regarded as a good index of increased adrenocortical activity. However, the relationship of adrenal ascorbic acid concentration to adrenal steroid production is still a controversial subject(1-3). Though the literature intimates



EXPERIMENTAL GROUP		PLASMA GLUCOSE (MG %)[4]
NORM. INTACT (14)	A	87±6
" + ALLOXAN, (14)	B[2]	121±10
" + ALLOXAN, (11)	C[3]	144±9
NORM. INTACT, GLUCOSE I.V. (7)	D	87±5
NORM. + ALLOXAN, GLUCOSE I.V. (6)	E	193±24

STATISTICAL ANALYSES OF DATA[5]

GROUPS	ADR. WT.	ADR. ASCB. AC.	PL. GLUC.
A−B	<.01	<.01	< 01
C−A	<.01	<.01	<.01
B−C	>.05	>.05	>.05
A−D	>.05	<.01	>..05
B−E	>.05	>.01	<.01

FIG. 1. Legend. 1—Bars represent mean ± S.E. 2—Members of this group received subcut. inj. of alloxan at dose of 150 mg/kg body wt. 3—Members of this group received intrav. inj. of alloxan at dose of 40 mg/kg body wt. 4—Mean ± S.E. 5—The probability of greater error occurring by chance of the difference between the means of the indicated groups. Figures in parentheses are the number of rats in each group.

that ascorbic acid depletion in the adrenal glands is a gauge of adrenocortical activity, this is probably true only in acute conditions. The relationship of gland content of hormone or precursor substance(s) to the factors of syntheses and discharge is still an unresolved problem which has been discussed in some detail by Sayers and Cheng(4). Since the functional relationship of adrenal ascorbic acid concentration to hormonal secretion is still unknown, experimental evidence of conditions in which adrenal ascorbic acid is increased is particularly noteworthy. Evidence will be presented here of an increase in both adrenal gland weight and adrenal ascorbic acid concentration in rats following alloxan administration.

Materials and methods. The data were obtained from the following experimental groups of male Wistar rats weighing 250-275 g: (A) Normal intact; (B) Normal rats approximately 65 hours after the subcutaneous injection of alloxan (5% solution of Alloxan monohydrate, Eastman No. 1722, 150 mg/kg body weight); (C) Normal rats approximately 65 hours after intravenous injection of alloxan (5% solution, 40 mg/kg body weight: (D) Normal intact 60 minutes after intravenous administration of 208 mg glucose; and (E) same as group B but 60 minutes after intravenous administration of 208 mg glucose. The glucose was administered as an 8% solution prepared in M/15 sodium phosphate buffer, pH 7.4. The rats were anesthetized with EVIPAL (n-methylcyclo-hexenyl-methyl barbituric acid) in preparation for either subcutaneous or intravenous injection of the different materials. The intravenous injections were done directly into an exposed saphenous vein. The adrenals were obtained from rats after an overnight fast. Adrenal ascorbic acid concentration was determined by the method of Roe and Keuther(5). Cardiac blood obtained by direct puncture was used in the determination of plasma glucose by the method of Kingsley and Reinhold(6).

Results. The mean ± S. E. adrenal weight (mg/100 g body weight) and the mean ± S. E. adrenal ascorbic acid concentration (mg/100 g fresh adrenal) of the several groups of rats are presented in Fig. 1. The probability of greater error occurring by chance of the difference between the means as determined from Fisher's "t" test for plasma glucose, adrenal weight, and adrenal ascorbic acid concentration are presented for the indicated groups below the bar graph. It is evident from an inspection of these data that the mean adrenal weight and ascorbic acid concentration of the two groups of alloxan-injected rats were significantly greater than the respective mean values of the normal intact group. Also, it is apparent that the mean adrenal weight and ascorbic acid concentration were not different in the two alloxan-injected groups although the route of administration of this substance was different.

The data for Groups D and E were obtained from analyses of adrenals of the normal intact and alloxan-injected (subcutaneous) rats 60 minutes after an infusion of 208 mg glucose in order to determine the comparative effect of an acutely stressful condition upon ascorbic acid depletion. Application of the Fisher's "t" test to the difference between the means of Groups A-D and Groups B-E show that there was no difference in the mean adrenal weight of these groups; but there was a significant depletion of adrenal ascorbic acid concentration after the glucose infusion in both the intact group (D) and the alloxan-injected group (E). It is noteworthy that the per cent of ascorbic acid depletion in both groups after intravenous glucose was relatively the same, 28% and 33% respectively.

Discussion. The data presented in Fig. 1 illustrate the acuteness of the problem of whether the concentration of adrenal ascorbic acid may be considered a reliable guide of the hyper- or hypo-activity of the adrenal cortex insofar as steroidal hormone secretion is concerned. The increased weight of the adrenal glands in the alloxan-injected groups indicate an adjustment to increased adrenocortical requirements in these rats since it is an established fact that the C-11 oxysteroids of the adrenal are in the main physiologically responsible for the maintenance of the increased blood sugar level in depancreatized and alloxan-diabetic animals. That the heightened concentration of adrenal ascorbic acid in the alloxan-injected groups in this study is not possibly the resultant of a decreased ability to release (discharge or synthetic incorporation) of this material is indicated by the fall in concentration of the ascorbic acid in Groups D and E after glucose infusion. The change in adrenal ascorbic acid concentration in these two groups was in accord with the accepted pattern of ascorbic acid depletion in response to an acutely stressful condition.

An explanation of the increased adrenal ascorbic acid concentration in the alloxan-injected groups can not be made from the data in this report. However there is evidence in the literature relating to the interrelationship between alloxan and glutathione on the one hand, and between ascorbic acid and glutathione which provide a promising speculative basis for an explanation of the results presented here. It has been shown that glutathione protects rats against alloxan diabetes(7). It also has been demonstrated (8-10) that following the injection of alloxan there is a fall in the glutathione content of tissues. The existence of an inverse relationship between ascorbic acid and glutathione is indicated by reports that ascorbic acid injection decreased the glutathione content of the blood(11) and the injection of glutathione decreased the ascorbic acid content of adrenals (12).

The relationship through glucose metabolism of the endocrine function of the pancreas and the adrenal glands is established. However, the effect of experimental diabetes on the function of the adrenal cortex and related constituents of this gland has not received the consideration that it deserves. Until further experiments along these lines are performed, the results presented here are of undoubted interest for investigators in this field.

Summary. The mean adrenal weight/100 g body weight, and the adrenal ascorbic acid concentration of groups of rats approximately 3 days after subcutaneous or intravenous injection of alloxan were shown to be significantly greater than the respective mean values in intact rats. The extent of adrenal ascorbic

acid depletion 60 minutes after subjection to an acutely stressful condition (208 mg glucose solution by intravenous administration) in intact and alloxan-injected groups was practically identical; and significantly lower concentration than control values were obtained. The results were discussed as further evidence of the problem whether adrenal ascorbic acid concentration may be considered a reliable guide of hyper- or hypofunction of the adrenal cortex. A tentative explanation of the increased ascorbic acid concentration in the alloxan-diabetic groups was also discussed.

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Effect of Vitamin C Deficiency on Healed Wounds.* (20034)

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The importance of vit. C in wound healing is well known. In advanced ascorbic acid deficiency wound healing is impaired(1-8). This impairment is characterized histologically by an abundant proliferation of fibroblasts which remain immature, by failure of formation of extracellular material, and by a marked reduction of histochemically detectable alkaline phosphatase. The small amount of extracellular material which may be produced in less severe cases does not become organized into collagen fibers, and argentaffine reticulum fibers (pre-collagen) persist. In addition, the new capillaries which form in this abnormal granulation tissue are defective, and lead to hemorrhages in the wound area. The tensile strength of the wounds is markedly reduced. The subject has been extensively reviewed by Bicknell and Prescott(8) and Levenson *et al.* (9). Vit. C has also been reported as essential for the maintenance of scar tissue even when this had formed many years previously. In Walter's account(10) of Lord Anson's voyage around the world (1769) it is stated that, "the scars of wounds which had been for many years healed were forced open again by this virulent distemper" (scurvy, editor). From this description it would appear that wound scar tissue is particularly susceptible to a prolonged ascorbic acid deficiency. Experimental proof of this susceptibility of scar tissue has been reported and commented upon briefly by Hunt(11) and Hartwell and Stone (12). On the other hand Robertson(13), in a recent study, measured chemically the amount of collagen present in healed wounds using the method of Lowry *et al.*, but failed to observe a significant reduction of its concentration in scorbutic guinea pigs.

It is the purpose of this study to present and discuss the nature of the gross and histologic changes taking place in healed wounds during ascorbic acid deficiency. The altera-

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* The opinions expressed in this paper are those of the authors and do not necessarily represent the official views of any governmental agency.

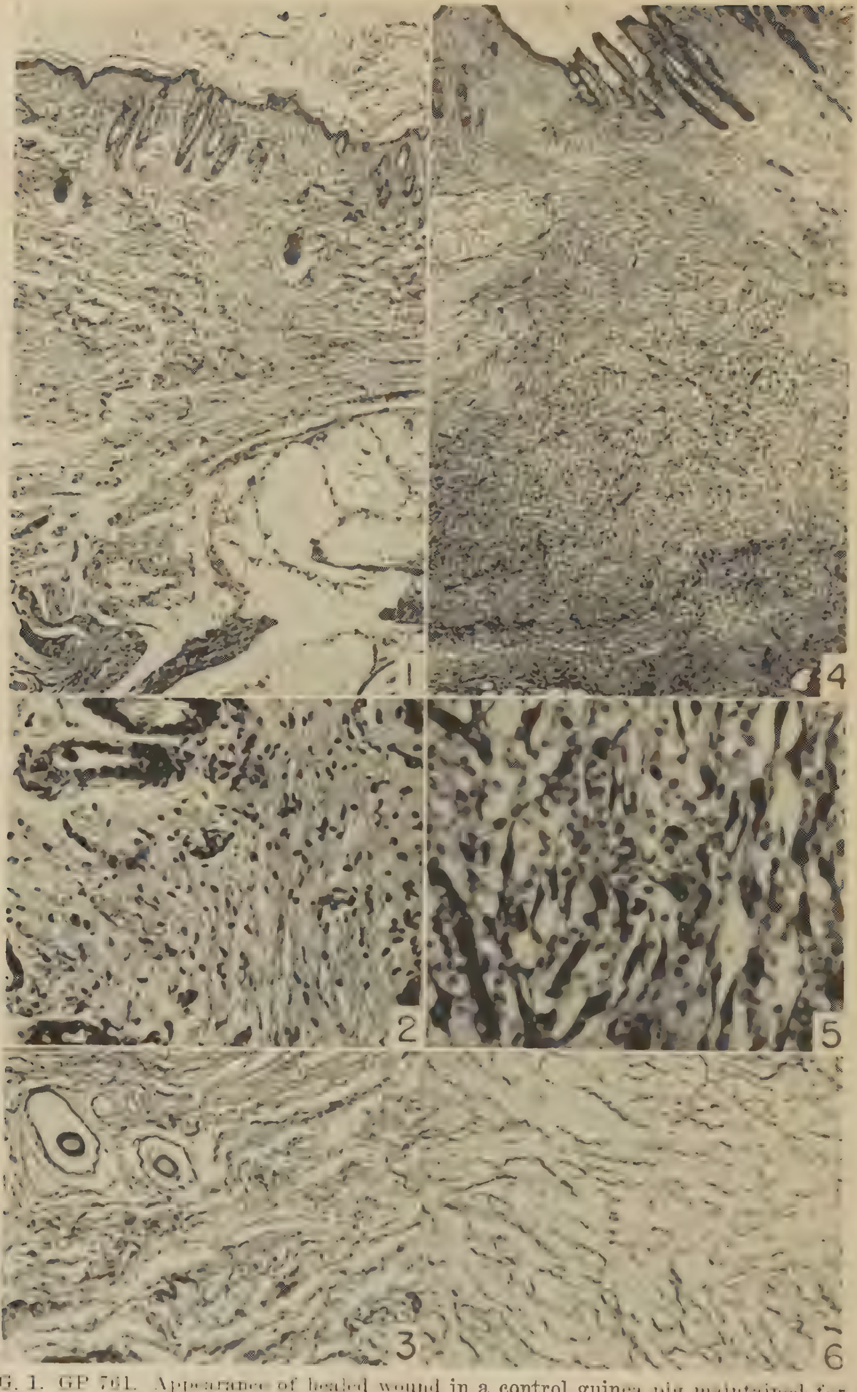

FIG. 1. GP 761. Appearance of healed wound in a control guinea pig maintained for 6 wk postoperatively on a complete ration and then pair fed for 20 days with a scorbutic animal. The scar consists of a thin band of connective tissue recognizable by the absence of accessory skin structures. H and E, × 50.

FIG. 2. GP 761. Detail of Fig. 1, showing broad, well-formed, collagen bands and a relatively small number of fibrocytes. H and E stain, × 265.

FIG. 3. GP 761. Argentaffine black reticulum fibers are few and scattered. Foot's stain, × 200 (approx.).

FIG. 4. GP 743. Appearance of the wound in a guinea pig maintained for 6 wk postoperatively on a complete ration and then fed for 26 days a diet totally deficient in ascorbic acid. The scar is replaced by abundant immature granulation tissue containing numerous capillaries and small hemorrhagic areas. H and E, × 50.

FIG. 5. GP 743. Detail Fig. 4. The granulation tissue consists of numerous immature fibroblasts separated by extravasated red blood cells and a few collagen fibers. Few defective capillaries can be recognized (left upper corner). H and E, × 500 (approx.).

FIG. 6. Numerous argentaffine reticulum fibers are present in many areas. Foot's stain, × 250 (approx.).

tions observed are consistent and characteristic.

Methods. Young adult male guinea pigs about 10 weeks of age and weighing on the average 450 g were placed in single wire-screen bottom cages and adjusted to laboratory conditions for about a week. They were fed ascorbic acid-free pellets† supplemented by adequate greens and fresh tap water *ad libitum.* A linear midline laparotomy wound, 4 cm in length, was then performed in each animal under nembutal anesthesia (3 mg/100 g body weight, intraperitoneally). Clean, but not aseptic, technic was employed. The wound was closed in one layer by 4 or 5 through and through fine stainless steel sutures which were removed 2 weeks later. The wounds were allowed to heal for 6 weeks, the animals being kept on a nutritionally complete diet. Following this period the guinea pigs were divided in 3 groups: Group 1 (13 animals "deficient") was fed the pellet ration *ad libitum* without supplements; Group 2 (6 animals "pair-fed controls") was pair-fed with an equal number of animals of Group 1, and received in addition 2.5 mg ascorbic acid per day orally; Group 3 (7 animals "*ad libitum* controls") was fed *ad libitum* the pellets plus 2.5 mg per day of ascorbic acid. When the guinea pigs of the first group had developed clinical signs of severe scurvy (average 26 ± 3 days) representative animals of all groups were sacrificed by exsanguination under nembutal anesthesia. Complete autopsies were performed. The wounds were resected immediately after death and fixed in 10% neutral formalin. Blocks from the center of the wound were embedded in paraffin. Sections cut at 6 μ were stained with hematoxylin eosin, Van Gieson (collagen), toluidine blue (acid mucopolysaccharides) and Foot (reticulum) technics. Small blocks of the wounds were also fixed in cold 80% alcohol for alkaline phosphatase. Details of histological methods used are given elsewhere (14).

Results. All guinea pigs gained on the average 40% in body weight in the 6 weeks following operation and were in excellent condition at the beginning of the experimental period. During the following 26 days of experimental regimen the scorbutic animals lost on the average 23% and their pair-fed controls 12.5% of their body weight, while the *ad lib.* controls gained 19%. The food consumption of the ascorbic acid deficient guinea pigs and of the pair-fed controls was on the average 32% less than that of the *ad lib.* animals during the same period. Obvious signs of scurvy developed in the majority of the "deficient" animals after 17 to 18 days and were apparent in all of them about 21 to 22 days following the withdrawal of ascorbic acid. At the time of sacrifice (26 days) the degree of scurvy was severe, although most of the animals were not in a terminal condition.

Gross findings. During the first 6 weeks the wounds of all animals healed normally. At the end of this period the wound scar was reduced to a thin line which was considerably shorter than the original wound and in many instances was hardly visible. Approximately 3 weeks following the withdrawal of ascorbic acid from the diet, changes were observed in many of the scorbutic animals. These consisted of swelling of the wound accompanied by a few small hemorrhages. Herniation of the wound developed in 3 cases. However, complete disruption did not occur and the epidermis appeared intact in all cases. No changes were noted in the wound scars of the *ad libitum* and pair-fed controls.

Microscopic findings. In both groups of control animals the wounds appeared entirely normal. The epidermis appeared somewhat thinner than that of the adjacent areas with flat or absent projections. The underlying connective tissue had a normal appearance consisting of rather thick fibers staining brilliant red with Van Gieson and a relatively small number of fibrocytes. Hyalinization was slight. Reticulum fibers were few in numbers (Fig. 1, 2, 3) and metachromasia was minimal. No sites of alkaline phosphatase activity were detected except at the periphery of a few hair follicles.

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† Rockland guinea pig diet especially prepared *without ascorbic acid* by the Arcady Mills Co., Chicago.

In the scorbutic guinea pigs the epidermis in the wound area appeared essentially similar to that of the controls and did not seem to be affected in any way by the deficient diet. In all cases the underlying connective tissue showed distinct changes. These changes varied considerably in severity from animal to animal, and were generally in accord with the severity of scurvy as judged by the gross and microscopic lesions of bones and joints. The changes were in the main limited to the immediate wound area extending laterally only for a short distance. The connective tissue appeared somewhat loose and was much more cellular than that of the controls. A large number of fibroblasts, immature mesenchymal cells and a few fibrocytes were present (Fig. 4). As a result, there was rather abundant granulation tissue in the wound area. This contained numerous capillaries some of which appeared to be defective and there were several areas of hemorrhage (Fig. 5). In no case did the collagen fibers stain brilliant red with Van Gieson's technic; the color, instead, varied from pale red to pink and, in a few animals, to either a diffuse or patchy yellow-pink color. Reticulum was present in much greater amount than in the controls and appeared either as a fine network or as groups of coarse irregular fibrils (Fig. 6). The degree of metachromasia, as judged by the toluidine blue stain, was markedly increased when compared to that of the controls. It was felt that this was due to an enhanced reactivity to toluidine blue rather than to an increased amount of mucopolysaccharides, probably on the basis of depolymerization of the ground substance which is thought to occur in scurvy as well as in other conditions (15,16). No alkaline phosphatase activity could be detected in the wounds of these animals. As a rule, a thin layer of connective tissue immediately beneath the epidermis showed either minimal or no regressive changes.

Discussion. The changes which develop in the connective tissue of a *healed* wound when ascorbic acid is completely removed from the diet are of the same type as those which occur in *healing* wounds of scorbutic animals and man. The appearance of a large number of immature mesenchymal cells which replace the relatively few fibrocytes, and the reversion of mature or immature collagen fibers simultaneously with reappearance of argentaffine reticulum and depolymerization of the ground substance, can be explained on the basis of our knowledge of the fundamental phenomena of scurvy. In this disease similar regressive changes occur in bone and in other tissues of mesenchymal origin, particularly where mechanical stress is applied as in muscles, tendons, cardiac valves, etc.(2,17,18). A thin layer of connective tissue immediately under the healed epidermis showed minimal changes. This can possibly be explained either by the very close approximation of the wound margins superficially which resulted in the formation of little granulation tissue in this area during the process of healing or perhaps by a supporting action of the epidermis, which was not visibly affected by ascorbic acid deficiency.

It is difficult to explain satisfactorily why a deficiency of vit. C should markedly affect normally healed granulation tissue (scar tissue) while it does not produce significant morphologically detectable changes in the adjacent normal connective tissue. There are however, well known definite gross and histologic differences between the 2 types of connective tissue: "old" scar tissue is less elastic, less vascularized, relatively acellular and contains denser collagen which frequently undergoes hyalinization. Although normal scar tissue appears morphologically "stronger" and has a tensile strength identical or greater to that of normal connective tissue, it is apparent that it is more susceptible to changes when subjected to metabolic stress. Our results clearly indicate that scar tissue is more sensitive than normal connective tissue to the lack of vit. C. The effect of vit. C deficiency is probably not mediated through a diminution of histochemically detectable alkaline phosphatase since a healed wound does not exhibit any stainable activity of this enzyme. In this respect an old scar differs from young granulation tissue. Our findings are in fundamental agreement with the recent studies of Gould and Gold(19) and throw considerable doubt on the importance of alkaline phosphatase in the wound healing process in general and on the maintenance of mature collagen in particular. A healed wound because of its retraction is probably subject to greater mechanical stress (traction from adjacent healthy tissues) and this may explain at least in part the type of changes observed. The relatively young age (6 wk) of the healed wounds studied may also have been a factor. In this respect it would be of interest to investigate whether older scars behave in a similar fashion. The presence of a normal concentration of collagen in healed wounds of scorbutic guinea pigs reported by V. B. Robertson(13) is not inconsistent with the histologic features previously described since such measurements obviously do not rule out the possibility of physical or architectural alterations in wounds.

Summary. When young adult guinea pigs bearing linear laparotomy wounds which had healed under normal conditions for 6 weeks were subjected to an ascorbic acid-free dietary regimen for approximately 26 days, severe changes were observed in the scar tissue area. The changes consisted of fibroblastic proliferation, regression of connective tissue elements, and of hemorrhages. The wounds of pair-fed and *ad lib.* control animals presented normal healing. The results indicate that adequate ascorbic acid intake is required not only for normal healing in the early post wound period but also for the maintenance of the scar tissue which has formed in healed wounds over a period of many weeks.

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Bovine Amniotic Fluid as Tissue Culture Medium in Cultivation of Poliomyelitis and Other Viruses.* (20035)

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Following the demonstration(1) that poliomyelitis virus could be propagated *in vitro* in cultures of human extraneural tissues a variety of cell types both of human and simian origin and of media for the support of these cells have been used by different investigators(2-8). In this laboratory human tissues have been largely employed and experiments have been continued with the purpose of developing more convenient and satisfactory procedures for the preparation and maintenance of large numbers of roller tube cultures. In particular we have been interested in tissues such as uterus that may be obtained in any large hospital as a by-product of surgical operation. Cultures of mature tissues have for some time been maintained with a medium consisting of balanced salt solution, ox serum ultrafiltrate, normal horse serum and beef embryo extract(9). In an attempt to reduce the number of constituents of this medium and with the hope of improving the growth of cells and of virus bovine amniotic fluid was substituted for the balanced salt solution and serum ultrafiltrate. The results of experiments will be briefly presented in which cell outgrowth and viral multiplication in these two kinds of medium were compared.

Materials and methods. The technics and materials adopted previously for the cultivation of mature human tissues in roller tubes have been described elsewhere(9). Bovine amniotic fluid was withdrawn from the amniotic sac in the following manner. The gravid uterus was removed *in toto* at the abbatoir and conveyed to the laboratory within 2 to 3 hours. It was suspended from a support by a cord. After cleansing and drying the entire surface of the organ, an appropriate site near the embryo and close to the dependent area was selected and lightly cauterized with a free flame. Taking care to avoid piercing the embryo, a special trochar (bore 1.5 mm, length 22.5 cm)† was then passed through this portion of the uterine wall into the amniotic cavity. The clear fluid which usually ran rapidly and freely was collected in sterile flasks. The yield of fluid varied from about 0.5 to 1.5 liters depending upon the size of the embryo. Occasionally fluids were contaminated with blood, presumably because of injury to the embryo by the trochar. These were not used for the preparation of culture medium. Embryos about 7.5 to 25 cm in length were selected. After withdrawal of the fluid the embryo was removed under aseptic precautions and its tissues employed for the preparation of embryonic extract(9). To the fluid withdrawn from each embryo sufficient penicillin and streptomycin were added to yield a final concentration of 50 units and 50 micrograms respectively per ml. Portions of the fluid (0.1 ml) were then added to thioglycollate broth and incubated for 48 hours to determine the presence or absence of bacteria insensitive to these antibiotics. In the meantime the fluids from each embryo were kept at about 6°C in tightly stoppered flasks. A pool was then made of the fluids from several embryos. Sufficient stock phenol red solution (0.4%) was added to the pooled fluid to yield a final concentration of 0.002%. The material was stored in the ice box. On storage the pH of certain batches was found to attain a high level (*e.g.*, 8.) This was reduced to 7.5-7.6 by "gasing" with a mixture of 5% CO_2 in air just before use.

Experimental. Effect of amniotic fluid on migration and growth of human cells deriving from mature uterine and renal tissues and from embryonic skin-muscle. In a number of experiments the growth of cells from human uterine tissue in roller cultures maintained with bovine amniotic fluid (BAF) alone or with the addition of beef embryonic extract

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* Aided by a grant from The National Foundation for Infantile Paralysis.

† Macalaster Bicknell Co., Cambridge, Mass.

TABLE I. Outgrowth in Roller Tube Cultures of Human Uterine Tissue Maintained with Various Media Containing Bovine Amniotic Fluid (Exp. 1).

Medium	Cell outgrowth on indicated day of cultivation					Remarks
	5	8	11	13	15	
BAF–100[*]	1[‖]	2	3	4	3	Slight retraction of coagulum
80, HS–20[†]	<1	2	2	3	3	Moderate to marked retraction
90, BEE–10[‡]	2	3	4	>4	>4	No retraction, cells granular
70, HS–20, BEE–10	3	4	>4	>4	>4	Moderate retraction
Regular med. (no BAF)[§]	2	3	4	3	3	Marked ”

[*] Bovine amniotic fluid–100%.

[†] Normal horse serum inactivated at 56°C for ½ hr–20%.

[‡] Beef embryo extract–10%.

[§] Regular medium: horse serum–20%, BEE–10%, Hanks-Simms' mixture–70%.

[‖] Numerals indicate mean outgrowth of cells in 6 comparable cultures estimated by simple inspection and recorded at 1+, 2+, etc.

(BEE) has been compared with the cell outgrowth in the "regular" medium hitherto used as routine for uterine tissue prior to inoculation of virus. The latter consisted of 10% BEE, 20% horse serum and 70% of a mixture consisting of 3 parts Hanks' balanced salt solution and 1 part bovine serum ultrafiltrate. No attempt has been made to determine by quantitative methods the amount of cell growth. A rough estimate that we regarded as sufficient for our purposes was obtained, however, by comparing in the various cultures at 2-day intervals the extent of cell outgrowth, its thickness (*i.e.*, one or more layers of cells) and the frequency of mitotic figures. The growth was thus evaluated as 1+ . . . . 4+ and >4+. Mean estimates of the outgrowth in 4 or 6 comparable cultures were calculated. The results of two experiments of this sort are summarized in Tables I and II. In the first experiment the medium was removed and replaced at intervals of 2 or 3 days; in the second the medium in one set of 4 cultures was removed and replaced at these intervals while in another set of 4 comparable cultures it was changed every 4 days.

The data of the first experiment indicate that BAF alone provides a medium in which certain cells of a tissue derived from a mature human being (age 40 years) not only survive but migrate from the original fragment and appear to increase considerably in numbers. These observations strongly suggest that the amniotic fluid not only provides a physiologic medium but contains a growth-stimulating factor or factors. It is evident, however, that BAF alone is incapable of inducing optimal cell growth since the addition of BEE and horse serum leads to greatly increased proliferation as indicated not only by the greater area and thickness of the zone of migrating cells but by the presence of large numbers of mitotic figures. When either BEE or horse serum is added to BAF, cell growth is enhanced, although BEE appears to be somewhat more effective. If employed without horse serum BEE favors the formation of granules in the cytoplasm. Accordingly it has seemed desirable to include horse serum as well in a medium for use as routine, since horse serum inhibits granulation when both these constituents are present in BAF. The cell growth in this medium was definitely more luxuriant than that observed in the so-called "regular" medium. Moreover, the cells nourished with the mixture containing BAF appeared to be in a more active condition as indicated by their larger size and the greater number of mitotic figures.

Although some retraction of the coagulum was noted in cultures containing BAF when horse serum was included, this did not seem to be so extensive as in those maintained with the regular medium. In this respect also the BAF appears to be an advantageous substitute for the balanced salt-serum ultrafiltrate mixture. Another useful property of BAF consists in the efficiency of its buffering action which is capable of preventing the pH from falling below 6.9-7 during a period of several days in actively growing cultures containing large amounts of tissue. The buffering action of BAF, therefore, is definitely more effective than that of the balanced salt solution-serum

TABLE II. Outgrowth in Roller Tube Cultures of Human Uterine Tissue Maintained with Various Media Containing Bovine Amniotic Fluid (Exp. 2).

Medium*	Medium changed	Cell outgrowth on indicated day of cultivation			Remarks
		6	11	15	
BAF–100	2†	1§	3	3	pH remained >7.5
	4‡	<1	3	3	
80, HS–20	2	1	4	4	Cells free of granules, very transparent —pH not <7.5
	4	1	3	4	
95, 5	2	1	4	4	
	4	1	3	4	
97.5, 2.5	2	<1	4	4	pH remained >7.5
	4	<1	2	3	
90, BEE–10	2	<1	4	4	Cells granular, pH lower than in cultures without BEE, *i.e.*, 7.2-6.9
	4	<1	3	4	
70, HS–20, BEE–10	2	1	4	>4	pH lower than in cults. with serum only, *i.e.*, 7.1-6.9 at time fluid changed
	4	2	4	>4	
85, 5, 10	2	1	4	>4	"
	4	2	4	>4	
90, 5, 5	2	2	4	>4	"
	4	1	4	>4	
87.5, 2.5, 10	2	1	4	>4	"
	4	1	4	>4	
92.5, 2.5, 5	2	1	4	>4	"
	4	<1	4	>4	
Regular medium (no BAF)	2	1	4	4	Retraction more marked than in cults. with BAF, pH dropped to 6.8 or lower
	4	2	4	4	

* For explanation of abbreviations see Table I.

† Fluids of 4 cultures changed Mondays, Wednesdays and Fridays.

‡ Fluids of cultures maintained with same medium but changed Mondays and Fridays.

§ Numerals indicate mean outgrowth of cells in 4 comparable cultures estimated by simple inspection and recorded as 1+, 2+, etc.

ultrafiltrate mixture. This property has permitted us to replace BAF medium in such cultures at intervals of 4 instead of 2 days as is necessary to maintain the pH above neutrality when the regular medium is employed. Thus, as the data included in Table II show, cultures nourished with BAF, BEE and horse serum and in which the medium is changed only at the longer interval exhibited continuing growth of cells which was essentially as luxuriant as that observed in cultures changed every 2 or 3 days. When large numbers of cultures are required, the saving in labor and materials achieved by adopting the 4-day interval for change of medium is considerable. In the presence of BAF variation in the quantities of horse serum and BEE within the range that has been studied has effected little or no discernible difference in cell growth as shown in Table II. We have, therefore, now adopted more or less arbitrarily but with consideration of economy of materials in mind a medium for routine purposes consisting of 5% BEE, 5% normal horse serum and 90% BAF. This medium has afforded consistently good growth not only of cells derived from human uterine tissue but also those from human kidney and prepucial tissues removed at operation and from a variety of tissues of the human embryo which include skin and muscle, liver, amniotic sac and umbilical cord (from embryos of 2-3 months gestation).

Multiplication of poliomyelitis virus in cultures maintained with BAF in the medium. Considerable experience has now been gained in the propagation of poliomyelitis viruses in cultures of various human tissues maintained with media containing BAF. The concentrations of virus developing in such cultures have equaled or exceeded those found in comparable cultures nourished with "regular" medium. Furthermore, the capacity of cultures maintained with BAF medium to support multipli-

TABLE III. Assay of Brunhilde Virus Grown in Roller Tube Cultures of Human Uterus Maintained with BAF and Regular Media and Titrated in Cultures Maintained with These Media.

Source of virus	Medium in cultures for titration	Dilution of virus inoculated 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
BAF culture*	BAF‡	1/1‖	2/2	2/2	2/2	0/2
Reg. med. culture†	BAF	1/1	1/2	1/2	0/2	0/2
BAF culture	Reg. m.§	1/2	2/2	2/2	0/2	0/2
Reg. med. culture	Reg. m.	1/1	0/2	1/2¶	0/2	0/2

* Pooled virus derived from 2 cultures of human uterine tissue maintained with BAF-85, HS-5, BEE-10 and each inoculated with 10 ID_{50} (tissue culture infecting doses).

† Pooled virus derived from 2 similar cultures maintained with Hanks-Simms' sol. 85, HS-5, BEE-10 and each inoculated with 10 ID_{50}.

‡ Cultures of human uterine tissue used for titration and maintained with medium mentioned in footnote 1.

§ Cultures of human uterine tissue used for titration and maintained with medium mentioned in footnote 2.

‖ Numerator: cultures exhibiting cell degeneration within 10 days following inoculation. Denominator: cultures inoculated.

¶ Cytopathogenic effect not evident until 9th day after inoculation; in all other cases this was apparent within 5 days.

cation of the virus following the introduction of minimal inocula has usually exceeded that of cultures with the regular medium. Illustrative data are presented in Table III which summarizes the results of an experiment in which the Brunhilde strain was first propagated in each of 2 cultures maintained respectively with the 2 kinds of medium. The virus suspensions thus produced were then titrated *in vitro*(3,9). It is apparent that in cultures with BAF medium the yield of virus as well as the end point of viral activity as denoted by cell degeneration were higher by a factor of about 10. It should be pointed out in respect to this experiment that somewhat higher yields of virus have frequently been achieved with the regular medium(9). However, in subsequent experiments with BAF medium evidence has been obtained that more virus developed than had previously been found in cultures maintained with the regular medium. The *greater sensitivity* of the cultures maintained with BAF in respect to the detection of small quantities of virus has also been apparent when the inocula have consisted of suspensions of feces from patients with poliomyelitis. In experiments with such materials carried out by Dr. Sidney Kibrick in this laboratory roller tube cultures supported with BAF medium were found to reveal the presence of approximately 1/10th the quantity of virus detectable by cultures containing "regular" medium. Moreover, in a reexamination of 24 10% stool suspensions from 24 patients from which no virus had previously been isolated in cultures maintained with regular medium, 5 agents were revealed through the use of cultures in which BAF medium was employed.

Multiplication of other viruses. Other viruses that include herpes simplex, the Boston strain of Coxsackie virus which displays cytopathogenic properties(10,11) and four agents isolated from fecal material which do not appear to belong to any of the known agents of this class(4) have been successfully propagated in cultures with BAF medium. These viruses as well as those of the poliomyelitis group have induced their cytopathogenic effects more clearly and promptly in cells growing in a mixture consisting of 90% BAF, 5% BEE and 5% horse serum.

Effect of BAF medium in cultures used for virus neutralization tests. Because of the possibility that BAF might in some way interfere with the neutralization of virus by specific antibody *in vitro* virus neutralization tests were done in parallel using cultures maintained respectively with the two types of medium. Three specimens of serum from a patient with poliomyelitis taken at various periods after the onset were tested. The results presented in Table IV suggest no interference by BAF with the action of antibody which was shown

TABLE IV. Neutralization of Brunhilde Virus by Serum of a Poliomyelitis Patient in Roller Tube Cultures of Human Uterine Tissue Maintained with BAF and Regular Media.

Serum of polio patient drawn*	Medium used in test cultures	Final serum dilution					
		1/8§	1/16	1/32	1/64	1/128	1/256
9/23/52	BAF†	0/2‖	2/2	ND	ND	ND	ND
	Reg.‡	ND	1/2	ND	ND	ND	ND
10/11/49	BAF	ND	ND	0/2	2/2	1/2	2/2
	Reg.	ND	ND	0/2	1/2	1/2	2/2
1/21/52	BAF	ND	ND	0/2	0/2	0/2	1/2
	Reg.	ND	ND	ND	0/2	0/2	1/2

* Specimens of serum obtained on dates noted from patient with paralytic poliomyelitis. Type I virus (Brunhilde) was isolated from feces of this patient during acute phase of disease.

† BAF = medium consisting of 5% horse serum, 10% beef embryo extract, 85% bovine amniotic fluid.

‡ Reg. = medium of 5% horse serum, 10% beef embryo extract, 85% Hanks-Simms' sol.

§ .1 ml of a mixture of diluted serum and virus added to each culture. 100 ID_{50} of Brunhilde tissue culture virus employed in each instance.

‖ Numerator: cultures exhibiting cell degeneration within 10 days after inoculation. Denominator: cultures inoculated.

ND = not done.

to increase in concentration with passage of time.

Discussion. The results presented here as well as much additional experience recently gained make it clear that BAF forms an excellent basis of a medium, when enriched by the addition of other materials, for the propagation *in vitro* of certain human cells. This finding is of value from the utilitarian standpoint since it has enabled us to obtain regularly excellent cell growth which has been accompanied by increase in the yield of poliomyelitis viruses as well as in the capacity of the tissue culture to reveal the presence of small quantities of these agents.

The observation that BAF alone without the addition of other factors promoting cell growth leads to a more restrained migration and proliferation of tissue cells is perhaps of general biologic interest. So far as we are aware the physiologic role of amniotic fluid has not been satisfactorily elucidated except that it may protect the embryo against injury induced by mechanical means. Although something is known concerning the chemical nature of the simpler constituents of the fluid(12,13), little or nothing has been recorded in respect to its possible content of certain biologically active entities such as vitamins, hormones or growth-promoting factors. The evidence for the presence of the latter which our investigation has afforded would seem to encourage attempts to define more exactly the biochemical properties of this interesting material. It is possible that the results of such studies might lead to the hypothesis that the amniotic fluid may represent at least one source whence the cells of the early embryo before the vascular system is established derive the materials for their maintenance and growth.

Summary. Bovine amniotic fluid induced active cell growth when substituted for balanced salt solution and bovine serum ultrafiltrate in a medium used for the cultivation of embryonic and mature human tissues in roller tube cultures. The growth was more luxuriant than that observed in comparable cultures maintained with the balanced salt mixture. Moreover, superior yields of poliomyelitis viruses have been obtained in cultures nourished with bovine amniotic fluid as a constituent of the medium and in such cultures the minimal infecting dose of these agents has also proved to be smaller. Bovine amniotic fluid does not interfere with the neutralization of poliomyelitis virus by specific antibody. Through the use of bovine amniotic fluid the labor and expense involved in the preparation and maintenance of tissue cultures applied to the propagation of poliomyelitis virus and certain other agents of this class have been reduced.

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Effects of Cortisone Acetate on Fluid and Electrolyte Balance in Normal Rabbits.* (20036)

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Although the effects of cortisone on the metabolism of sodium, potassium and water have been extensively investigated in diseased man and in the normal rat and dog(1-4), comparable data in the rabbit are lacking. Most studies in immunology, however, have been performed in the latter species of animal. Because of the variability in the response of different species to drugs, a study of the effects of cortisone on fluid and electrolyte metabolism in the rabbit appeared to be desirable.

Recent studies have suggested that immune reactions in the rabbit might be associated with alterations in the distribution of body fluid and electrolytes, and that the adrenal gland may be involved in these changes(5,6). Before the role played by the adrenal gland in the phenomena of immuno-physiology can be interpreted, it is first necessary to determine the effects of adrenal hormones in normal animals. The availability of radioactive potassium (K^{42}) has made possible the direct measurement of the exchangeable potassium content in intact animals. The purpose of the present study was to determine the effects of two different dosages of cortisone acetate on sodium, potassium, and water metabolism in normal rabbits.

Materials and methods. Fourteen normal adult male rabbits of mixed breeds were used. They were kept in individual metabolism cages and were fed a stock diet of compressed pellets. Tap water was given without restriction. In order to acclimate the animals to their new environment, they were placed in the cages one week prior to the initiation of the baseline studies. *Procedure.* Two groups of 7 rabbits were used. External balance studies of sodium and potassium were performed daily for 4 weeks in Group 1 and for 5 weeks in Group 2. Direct measurement of the exchangeable potassium content (Ke) was made with K^{42} at weekly intervals. During the first week, baseline studies were done on each group. Starting on the 8th day

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and continuing every day for 2 weeks, 4 mg of cortisone acetate suspended in one ml of physiologic saline solution was given subcutaneously in the interscapular space to each animal in Group 1. This dose is roughly comparable to that given therapeutically (100 mg daily for a 70 kg man). Starting on the 8th day and continuing every day for 3 weeks, 25 mg of a suspension of cortisone acetate containing 25 mg/ml was given in the interscapular space to each of the 7 animals in Group 2.

External balance of sodium and potassium. Each morning before the rabbits were fed, the body weight, total food consumption, and 24-hour urine volume were measured. Each gram of feed contained 0.275 meq of potassium and .09 meq of sodium. The tap water contained a negligible amount of both ions. The potassium and sodium content of the ingested feed was found to be completely absorbed through the gastrointestinal tract; the amount of these ions in the stool was too low to be detected by flame photometry. The weekly net gain or loss of sodium and potassium from the body, therefore, was calculated on the basis of the difference between the amounts ingested and those excreted in the urine. *Measurement of exchangeable potassium content.* The initial Ke determination was made on the first day of the experiment; the second, on the 7th day. The mean of these 2 determinations was used as the baseline value for each animal in the analysis of the data. Subsequent Ke determinations were made at weekly intervals. The preparation of the potassium solution for injection has been previously described(7). Five ml of the potassium solution, containing 30 μc of K^{42} in 0.5 meq was injected intravenously. The urine was collected for 24 hours; a spot specimen was then obtained by catheterization and its specific activity was determined. The following formula was used to calculate the value for the exchangeable potassium content (Ke) of the body:

$$Ke = \frac{Ki^{42} - Ko^{42}}{Ku^{42} / Ku^{39}}.$$

Ki^{42} = quantity of radiopotassium administered (arbitrary units).

Ko^{42} = quantity of radiopotassium excreted in urine in 24 hr.

Ku^{42} = concentration of radioactivity in the spot specimen.

Ku^{39} = concentration of total inorganic potassium in the spot specimen.

Ku^{42} / Ku^{39} = specific activity of the spot specimen.

The potassium concentration in the urine and in the serum was determined by flame spectrophotometry by a method which has been described previously(8).

Statistical method. The significance of the difference between the means was determined by the use of the "t" test(9). Each animal served as its own control. The differences between the baseline value and the values at the end of the second, third, and fourth weeks in Group 1 (and also at the end of the fifth week in Group 2) were calculated for each animal; the mean difference between the baseline values and each of the subsequent determinations was then obtained. The significance of the mean difference was tested by the following formulae in which d = mean difference, s_d = standard error of the mean difference, S = summation of, and n = number of animals:

$$t = \frac{d}{s_d} \qquad s_d = \sqrt{\frac{Sd^2 - \frac{(Sd)^2}{n}}{n(n-1)}}$$

The calculated value for "t" was compared with the value at (n—1) degrees of freedom, as obtained from standard tables, and expressed as "P". A "P" value of 0.02 or less was considered significant.

Results. Group 1. The daily administration of 4 mg of cortisone acetate resulted in the significant changes shown in Table I. After the administration of cortisone *for 7 days,* an increase in body weight and a reduction in serum sodium concentration had occurred. After *14 days* of cortisone therapy, the mean serum sodium and potassium concentrations were significantly greater than their mean baseline values. The animals voluntarily reduced their intake of sodium although no change in sodium balance occurred. One week after the administration of cortisone was *discontinued,* the animals were in negative sodium balance, primarily because of a reduction in intake. There was no change in potassium balance, although the concen-

TABLE I. Effects of Cortisone Acetate, 4 mg Daily, on Fluid and Electrolyte Balance in Normal Rabbits.

	Wt (kg)	K intake (meq/day)	K excretion (meq/day)	K balance (meq/day)	Na intake (meq/day)	Na excretion (meq/day)	Na balance (meq/day)	Serum Na (meq/l)	Serum K (meq/l)	Exchangeable K (meq)	Exchangeable K (meq/kg)
Baseline values	3.17	34.34	29.24	+5.10	13.32	10.02	+3.20	138.3	4.3	199.4	62.6
Week 1†	+.16*	+5.89	— .03	+5.92	+ .10	—.15	+ .25	—9.9*	+ .5	+13.4	+ .7
2‡	+.12	+ .53	—2.92	+3.45	—1.84*	—.19	—1.65	+7.3*	+ .5*	—25.9	—13.8
3§	—.03	—8.89	—7.05*	—1.84	—4.90*	+.38	—5.48*	+2.8	+1 *	—24.7	— 3.2

* Statistically significant difference when this figure is compared with the baseline value; P = <.02.
† Mean changes after 7 days of cortisone. ‡ Mean changes after 14 days of cortisone. § Mean changes 7 days after discontinuing cortisone.

TABLE II. Effects of Cortisone Acetate, 25 mg Daily, on Fluid and Electrolyte Balance in Normal Rabbits.

	Wt (kg)	K intake (meq/day)	K excretion (meq/day)	K balance (meq/day)	Na intake (meq/day)	Na excretion (meq/day)	Na balance (meq/day)	Serum Na (meq/l)	Serum K (meq/l)	Exchangeable K (meq)	Exchangeable K (meq/kg)
Baseline values	2.40	46.04	19.85	+26.19	15.07	3.51	+11.56	146.2	5.2	168.9	70.5
Week 1†	+.06	—13.95*	+4.53*	— 5.55*	—4.56*	+ .99	—18.48*	+4.26*	— .73	—11.90	—6.95
2‡	- .10	—19.79*	+4.52	— 7.59*	—6.48*	+1.11	—24.31*	+4.84	— .49	—18.61	—5.46
3§	—.34*	—22.91*	+8.11*	—10.12*	—7.49*	+2.62*	—31.02*	+7.11*	—1.11	—30.55*	—3.56
4‖	—.31*	—22.47*	—1.61	— 7.45*	—7.35*	— .10	—20.86*	+4.21	+ .08	—35.88	—7.32

* Statistically significant difference when this figure is compared with the baseline value; P = <.02. † Mean changes after 7 days of cortisone.
‡ Mean changes after 14 days of cortisone. § Mean changes after 21 days of cortisone. ‖ Mean changes 7 days after discontinuing cortisone.

tration of potassium in the serum was increased.

Group 2. The daily administration of 25 mg of cortisone acetate resulted in the significant changes shown in Table II. After the administration of cortisone for *7 days,* the animals were in negative potassium balance, with an increase in the urinary excretion and a decrease in the oral intake of this ion. Sodium intake was decreased and the animals were in negative sodium balance. The mean serum sodium concentration was elevated. After *14 days* on cortisone, potassium and sodium balances were negative and the intake of both ions was spontaneously reduced. After *21 days,* a significant reduction in body weight had occurred. Potassium and sodium balances were still negative, and there was a further increase in the urinary output of both ions and a decrease in their oral intake. The serum sodium concentration was increased still more. Ke was significantly lower than the baseline value. After cortisone had been *discontinued* for a week, body weight was still significantly reduced below the baseline value. Sodium and potassium balances were negative, and the reductions in the intake of sodium and potassium were still significant.

Comment. The results of the present study indicate that the response of normal rabbits to cortisone acetate is dependent both upon the dosage and upon the length of the period of administration. Four mg of cortisone acetate produced at the end of the first week an increase in body weight and a decrease in serum sodium concentration, suggesting a retention of water and dilution of the extracellular fluid phase. The increase in serum sodium and potassium concentrations at the end of the second week indicate hypertonicity of the extracellular fluid space, which in turn suggests overhydration of tissue cells. One week after discontinuation of cortisone, loss of water and sodium through diuresis had produced a reduction in body weight and a negative sodium balance. Although the serum potassium concentration was elevated, no significant change occurred in the potassium balance or in the exchangeable potassium content. The results of the administration of this dosage of cortisone can, therefore, probably be attributed to changes in the distribution of water.

On the other hand, a daily dosage of 25 mg of cortisone acetate resulted in striking alterations in both sodium and potassium metabolism. After the first week of cortisone administration, the intake of both sodium and potassium was decreased (the animals were not eating) at the same time that the urinary excretion of potassium was elevated. The fact that no significant change in body weight occurred for the first 2 weeks, in spite of this negative potassium and sodium balance, suggests that there was a retention of water. Since the serum sodium concentration was increased at this time, this excess water must have been in the intracellular phase. The changes at 14 days were similar to those at 7 days. After 21 days on this dosage of cortisone, the body weight was decreased and the animals were in negative sodium and potassium balance. A significant reduction in exchangeable potassium content was detected for the first time, and the serum potassium concentration tended to be low. These changes had not been corrected 7 days after the administration of cortisone was stopped. Such changes suggest an increase in the rate of catabolism of tissues with an associated loss of water through the kidneys. It has previously been demonstrated in patients that the administration of either ACTH or cortisone in therapeutic doses may result in a depletion of the body content of potassium(10).

It would, therefore, appear that the earliest change to occur following the administration of a small dose of cortisone acetate to normal adult male rabbits is a retention of water in the extracellular fluid phase. As the administration of the drug is continued, the water is redistributed into the intracellular phase. The administration of a larger dose of cortisone results in depletion of the body's store of potassium, presumably because of the catabolic effects of cortisone as well as the decrease in food consumption. As the administration of cortisone is continued, diuresis eventually takes place.

The results of previous clinical studies dealing with the effects of cortisone on salt and water metabolism are difficult to interpret because of the differences in the physiologic status of the diseased subjects prior to the initiation of cortisone therapy. A fixed dose of the drug may not necessarily represent a physiologic dose in such individuals; furthermore, the response of the tissue cells to cortical hormone may vary with their functional status. Thus, different authors(12) have reported hyponatremia and hypernatremia respectively as the sole effects of cortisone therapy. Contrary to the findings in rabbits, large doses of cortisone have not produced alterations in the serum sodium or potassium concentration of rats, although such dosages did decrease food consumption(11). In dogs, the administration in doses of 5 mg/kg of cortisone daily for one week is reported to have caused a reduction in the serum sodium concentration(12).

The present study does not elucidate the mechanisms whereby alterations in the distribution and concentration of cations and water result from the administration of cortisone. However, it does show that rapid and transitory alterations in the distribution and osmolarity of body fluids may occur even in animals with intact adrenal glands in which hormonal imbalance is induced by the administration of cortisone acetate. The effects of cortisone on fluid and electrolyte balance in disease states must be interpreted in the light of the changes which it may induce in normal animals.

Summary. 1. External balance studies for sodium and potassium and serial isotopic determinations of the exchangeable potassium content were made in 2 groups of normal male rabbits which were given, respectively, 4 mg of cortisone acetate daily for 2 weeks and 25 mg daily for 3 weeks. The administration of 4 mg daily resulted in an increase in body weight and a reduction in serum sodium concentration within 7 days; both serum sodium and serum potassium concentrations were increased in 14 days. One week after discontinuation of cortisone, the animals were in negative sodium balance. The administration of 25 mg daily resulted in a negative sodium and potassium balance with an increase in serum sodium concentration within 7 days. At 21 days, body weight and exchangeable

potassium content were reduced. 2. The results indicate that the response of normal rabbits to cortisone acetate varies with the size of the dose and with the length of the period of administration. The results suggest that the earliest change to occur following the administration of a small dose daily was a retention of water in the extracellular fluid compartment; with continued administration of this dose, water was redistributed into the intracellular phase. The administration of a larger dose of cortisone daily resulted in depletion of the body's store of potassium.

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Effect of Salicylic Acid on Adrenal-Pituitary System.[*] II. Influence of Aminoacids and Other Compounds. (20037)

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The influence of salicylic acid (SAL) on the adrenal pituitary system of normal rats, as evidenced by a reduction of adrenal ascorbic acid, has been established by several investigators(1-5). This effect is quite specific for many substituted aromatic ortho-hydroxy carboxylic acids(5) and several other types of compounds(1,5). Since the metabolism of SAL involves conjugation with glycine and glucuronic acid, oxidation to gentisic acid and possibly other reactions, and since SAL bloodlevels can be influenced by administration of other substances, (PABA, ascorbic acid) (6-8), the effect of such combinations on the adrenal pituitary system was investigated. The main purpose was to determine whether it might be possible to augment the specific activity of SAL.

Methods. All experiments were made in female Wistar rats in the manner described previously(5). All compounds were administered as the sodium salts (except calcium pantothenate.) The pH of the solutions was not adjusted. The results for the treated animals are expressed in per cent of those for saline controls, since it had been shown that thereby the variations from day to day and from group to group are eliminated.

Results. The substances combined with SAL may be divided into 3 groups: carbohydrate metabolites, aminoacids and miscellaneous compounds. (Table I) None of these 21 acids, at a dose of 150 mg/kg, had any influence upon the ascorbic acid depletion produced by the simultaneous administration of 150 mg/kg of SAL. Particular attention was given to glucuronic and p-aminobenzoic acid because the former conjugates with SAL and both have been suggested for the treatment of rheumatoid arthritis and collagen diseases(9-12). PABA has also been shown to produce higher SAL bloodlevels by interference with renal clearance.

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TABLE I. Compounds Which in a Dose of 150 mg/kg Did Not Change the Effect of 150 mg/kg of Salicylic Acid on Adrenal Ascorbic Acid of Normal Rats. ( ) No. of animals.

Carbohydrate metabolites (acid)		Aminoacids		Miscellaneous compounds (acid)	
Acetic	(4)	d,l-α-Alanine	(4)	Adipic	(4)
Citric	(20)	β-Alanine	(8)	p-Aminobenzoic	(11)
Fumaric	(4)	l-Asparagine	(8)	Ascorbic	(4)
Lactic	(7)	l-Cystine	(8)	Gluconic	(8)
Pyruvic	(4)	l(-)-Leucine	(8)	Glucuronic	(28)
Succinic	(8)	d,l-Methionine	(8)	Pantothenic	(24)
		N-Phenylglycine	(8)		
		l-Tryptophane	(8)		
		l-Tyrosine	(8)		
		Hippuric acid	(4)		

TABLE II. Effect of Glycine upon Adrenal Ascorbic Acid Depletion Produced by Salicylic Acid. ( ) No. of animals.

	Dose in mg/kg			
	75	150	300	600
	Adrenal ascorbic acid in % of saline controls			
Salicylic acid	92(8)	78 (10)	61(12)	
Glycine			101 (4)	96(11)
Salicylic acid + glycine (equal parts)	86(7)	63* (20)		

* This value is significantly different from the corresponding SAL value. (p = .01.)

The only compound which has been found to augment the SAL action on adrenal ascorbic acid was glycine. (Table II). While this aminoacid alone, up to 600 mg/kg, had no effect, an amount of 150 mg/kg given together with an equal dose of SAL significantly increased the action of the latter on adrenal ascorbic acid. Even with smaller doses of both SAL and glycine, the results point in the same direction, but due to the small size of the absolute decrease in ascorbic acid concentration, the experimental variations are too great to make the difference statistically significant. Blood-level determinations of SAL did not show any change produced by glycine.

Discussion. The experimental data furnish additional evidence that the action of SAL on the adrenal pituitary system must be considered as a specific effect, quite different and distinguished from a general, nonspecific stress reaction. The mechanism by which glycine potentiates the SAL effect is not known. It is obvious from the experimental data that glycine has no direct influence, but acts only in a supporting role. A conjugation of SAL with glycine can also be excluded since salicyluric acid has been shown(5) to be much less effective than SAL in its influence on adrenal ascorbic acid. Friedman(13) had reported that the uricosuric action of SAL is also augmented by glycine. Both the depletion of adrenal ascorbic acid and urinary excretion of uric acid are assumed to be the result of an increased amount of circulating ACTH. Thus, the hypothesis is suggested for future investigations, that glycine either acts as a precursor or as a protector for ACTH, or that it inactivates through conjugation an ACTH neutralizing substance (cortisone?).

The observation that addition of p-aminobenzoic acid did not have any effect on the SAL action is due to the fact that the present experiments lasted only 2 hours. During this time, p-aminobenzoic acid has no appreciable influence on Sal bloodlevels. Analyses showed an average of 29.4 mg % of SAL as compared to 27.5 mg % when no PABA was given.

Summary. 1. The influence of aminoacids, carbohydrate metabolites and several other compounds on the adrenal ascorbic acid depletion produced by salicylic acid has been investigated. 2. Glycine is the only compound so far found which potentiated the action of salicylic acid. 3. This observation is an additional evidence in favor of a specific effect of salicylic acid on the adrenal-pituitary system.

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Morphologic Changes in the Dog's Adrenal Gland Following Anoxia.* (20038)

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Recently Selye(1) in his book on Stress summarized the literature on the adrenal gland. Apparently many observations have been made on the histochemical changes in the adrenal gland following periods of anoxia; however, few studies on morphology have been made. Rats exposed to chronic anoxia show hypertrophy of the cortex and a depletion of lipids in the zona fasciculata and zona reticularis(2-5). Since prolonged anoxia causes severe damage to the brain(6), it was of interest to ascertain whether the adrenal cortex also was damaged by anoxia. Because the adrenal gland has the greatest blood flow of any organ in the body, 5-8 ml/g/min.(7), it seemed likely that this reflected a high metabolism and that the gland therefore might be as susceptible as the cerebrum to anoxia. The present experiment gives the data obtained in this study, supplementing a previous preliminary report(8).

Experimental methods. The dogs were obtained from the pound and used soon thereafter. From their physical appearance their health was classified as excellent, good or poor. The method of producing anoxia and of resuscitating dogs has been described(9). Pure nitrogen was given unanesthetized animals to breathe by means of a special head mask. In this type of fulminating anoxia, the breathing failed within one to 2 minutes and the pulse pressure disappeared after 2 to 4 minutes. The dogs were resuscitated by two measures: periodic insufflation of the lungs with oxygen, using a special resuscitating valve for the head mask, and extrathoracic cardiac massage. This latter procedure was carried out with the dogs lying on their sides. The chest was compressed sharply between thumbs and fingers of both hands at a rate of approximately one hundred times per minute. This massage was stopped briefly every half-minute or so to ascertain whether the circulation had recovered. Soon after the blood pressure started to rise, respiration returned and quickly increased to normal pulmonary ventilation. During the first 24 hours following the induction of anoxia, episodes of decerebrate rigidity, running movements and ululation were common. The animals frequently flung themselves around the cage in apparent frenzy, with fits of "sham rage" often apparent. All grades of delirium and stupor occurred. The dogs then progressed into coma, and death occurred within two to three days. The few dogs that recovered did so slowly, showing ataxic attempts to stand and slow return of vision and hearing; after 4 or 5 days they appeared

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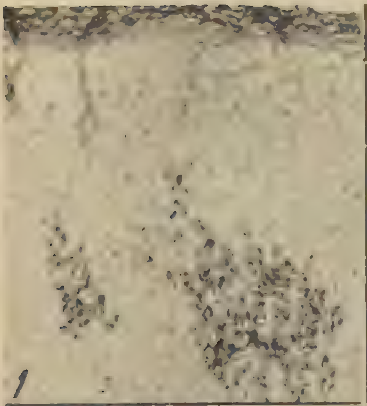

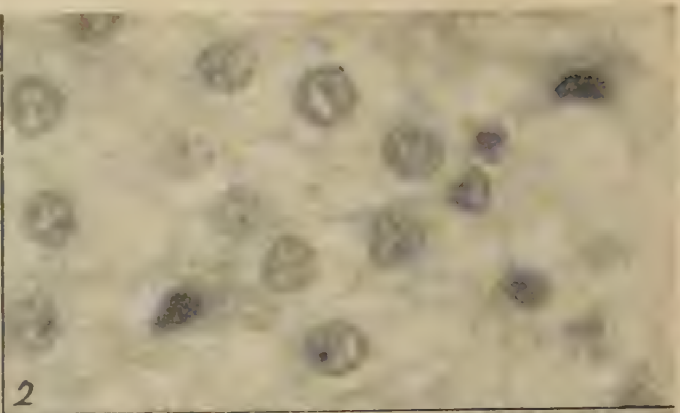

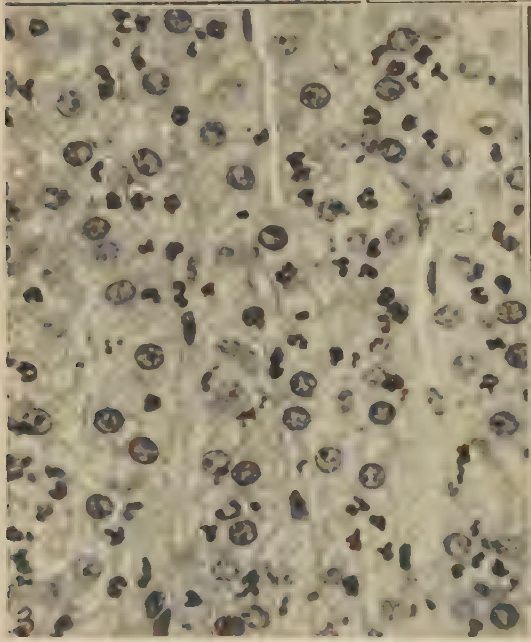

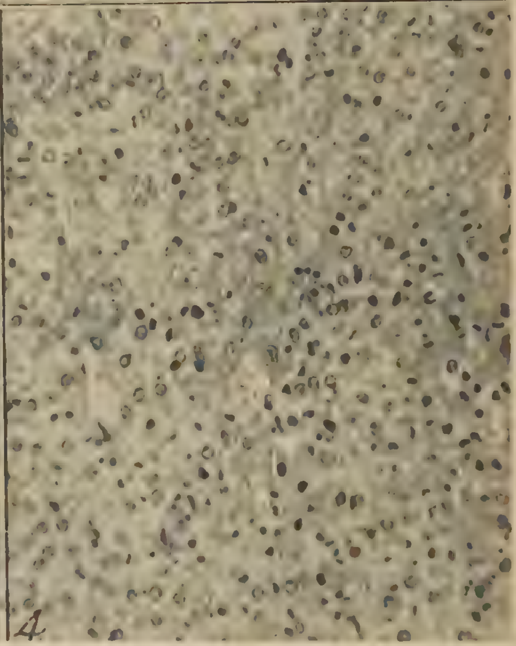

FIG. 1. Length of anoxia and interval before death for each dog is given in Table I. Dog 188, focal areas of hemorrhage in the cortex of the adrenal. Azo-Carmine stain × 75.

FIG. 2. Dog 196. Pyknotic nuclei and coagulated cytoplasm apparently are the minimal lesions occurring in the adrenal. H & P × 1026.

FIG. 3. Dog 172. Polymorphonuclear leucocytes frequently infiltrate the adrenal cortex of these anoxic dogs. H & P × 559.

FIG. 4. Dog 188. The pyknosis and coagulation necrosis occurring in the cortex may be diffuse. H & P × 440.

normal. All animals were given good nursing care; water and a starch solution were given by stomach tube. The ability to drink and eat spontaneously returned only after about 5 days. The method of classification of the clinical symptoms which we employed is slightly modified from that suggested by Delarue(10) from his studies on traumatic unconsciousness. These classifications are: 1) Conscious; 2) confusion; 3) delirium; 4) stupor; 5) semi-coma; 6) coma; and 7) death. Twenty-six dogs subjected to from 5½ to 8½ minutes of fulminating anoxia and 4 controls were used. They were sacrificed either by electric shock or exsanguination and autopsied immediately. All the viscera were examined macroscopically; however, only specific tissues were kept for histological study, except

TABLE I. Effect of Anoxia on the Adrenal Gland.

Animal No.	Physical state	Length of anoxia, min.	Interval before sacrifice, hr	Clinical manifestation when sacrificed	Degree pathologic change
206	Excellent	6.5	6	Confusion	0
207	Poor	"	"	Delirium	+
209	Excellent	"	"	Semi-coma	+++
205	"	"	24	Coma	+
208	Good	"	"	Stupor	+
210	"	"	"	Coma	+
102	Excellent	8	"	"	+
30	"	8.5	"	"	+
173	Poor	6.5	"	Death*	++
82–2	Good	8	"	Coma	++
82–3	Excellent	"	"	"	++
192	"	6.5	"	Semi-coma	+++
188	"	"	"	"	++++
172	Good	"	"	Coma	++++
99	"	8	"	"	++++
219	Poor	6.5	48	Stupor	0
220	Excellent	"	"	Confusion	0
14	"	"	"	Stupor	+
15	Good	"	72	Conscious	0
16	"	"	"	"	+
180	Excellent	"	96	Delirium	0
214	"	"	"	Conscious	0
215	"	"	"	Coma	0
174	"	"	"	Stupor	+
184	"	5.5	"	Conscious	+
196	"	6.5	120	Delirium	+

* Autopsied immediately after death.

for the adrenals which were carefully examined in every animal. Tissues for microscopic study were fixed in a dilute solution of formaldehyde; paraffin sections were prepared and stained routinely with hematoxylin and phloxine. Some sections were stained by Mallory's aniline blue method and by the periodic acid technic.

Experimental. The period of anoxia and the interval before death for each dog is given in Table I. At autopsy no attempt was made to determine the relative size of the adrenal glands since the animals were mongrels and varied much in size, weight and state of nutrition.

The adrenal gland on each side was similar. Dilated blood vessels and focal areas of macroscopic hemorrhage were present in the adrenals of a few dogs (Fig. 1). The earliest microscopic change occurred in the nucleus and cytoplasm of the epithelial cells in the zona fasciculata and zona reticularis. The nucleus in the injured cells was pyknotic and the cytoplasm appeared to be coagulated, staining deeply with phloxine (Fig. 2). The number of such injured cells varied; however, the lesion was diffuse. This cytological change is recorded as one plus in Table I. Polymorphonuclear leucocytes infiltrated the zona fasciculata and zona reticularis but were not found in the zona glomerulosa (Fig. 3). The number of leucocytes varied as indicated by 2+, 3+ and 4+ in Table I. Only occasionally were they found in groups. Usually the leucocytes were restricted to the zona fasciculata and zona reticularis; however, in three dogs (99, 172 and 173) they were also present in the medulla of the adrenal. Extensive necrosis was observed in only one dog, No. 188. This occurred in the zona fasciculata and zona reticularis and was a diffuse lesion. This necrosis was characterized by a coagulation process not associated with an abnormal number of phagocytic cells (Fig. 4).

The data in Table I are arranged to show the increased length of time between the period of anoxia and the time of death. It may be seen in this table that the maximum injury to the adrenal gland occurs in dogs sacrificed between 6 and 24 hours. Each of 12 dogs sacrificed at 24 hours showed pathologic lesions; only 5 showed a 1+ and 7 a

2+ lesion or more. Of the 11 dogs sacrificed after 48 hours, 6 did not show any pathologic changes in the adrenal and 5 showed only a one plus lesion.

The correlation between the degree of pathologic change and the clinical symptoms at the time of death is not close. Many animals that were comatose had only minor degenerative changes in the adrenals. These observations would suggest that death in this group of anoxic dogs is not the direct result of adrenal gland injury. Dog 215 was comatose at the end of 96 hours, but no lesions were found in the adrenals. There is nothing in this experiment to indicate that dogs sacrificed after 48 hours had had a more severe pathologic change during the earlier part of the experiment and the lesions then subsided. The cerebral damage resulting from the anoxia, no doubt, is a significant factor in the mechanism of coma and death in these animals.

Summarizing the clinical changes in 36 dogs made anoxic, 10 died (they are not included in the pathologic study). Of 24 made anoxic for 6½ minutes, 4 recovered or would have recovered if not sacrificed; of 6 made anoxic for 8 minutes, 4 were comatose when sacrificed for histologic study and 2 died; of 5 made anoxic for 8½ minutes, 2 were comatose when sacrificed for autopsy and 3 died.

Many of the dogs had pulmonary edema, petechiae and bronchopneumonia. A few had small lacerations in the liver. Sometimes the brain was edematous grossly; microscopically, extensive neurological changes were present in a majority of the animals. The pituitary and the thyroid glands were studied in many of these anoxic dogs but no significant morphologic changes were found.

Discussion. The morphological studies on the adrenal gland of anoxic dogs show changes that may be irreversible. However, as the gland has considerable power of growth and regeneration, this is not thought likely. The infiltration of polymorphonuclear leucocytes through the cortex is indicative of severe injury. It is of interest to observe that this injury occurs in the zona fasciculata and zona reticularis. No morphological changes were noted in the zona glomerulosa. Polymorphonuclear leucocytes were observed in the medulla of only 3 dogs and these animals also showed very severe lesions in the cortex.

In considering the pathogenesis of these adrenal lesions, it was originally thought that the effect might be secondary to a change within the pituitary gland. No morphologic changes were observed, however, in the pituitary gland of any dog. The facts that the adrenal gland has such a great flow of blood through it, that it apparently secretes such large quantities of hormone(11), and that it has been shown here to be susceptible to anoxic damage—all these facts appear to be related to one characteristic: that is the tissue is metabolically very active. Haldane (12) originally pointed out that anoxia not only slows the bodily machine, it causes irreparable damage to living structure as well. One would expect, therefore, that a tissue with high metabolic activity would suffer irreparable damage sooner than another tissue with low metabolic activity. The adrenal cortex follows this rule: its susceptibility to anoxic damage is apparently exceeded by only one other organ in the body, *viz.*, the brain. The clinical manifestations of anoxia in these dogs appear to be related primarily to the cerebral anoxia rather than to adrenal insufficiency. In support of this hypothesis, the clinical symptoms in four anoxic dogs were not affected by large doses of cortisone[†] given intramuscularly following the period of anoxia.

The variation in the degree of pathologic changes in the adrenals of dogs made anoxic for some length of time would suggest chemical differences within the glands. If the lesions could be inhibited, the agent might be of therapeutic value in cases of anoxia in man. It would seem necessary, however, to use other animals than mongrel dogs, as we have done, for such a study. The occurrence of pathologic lesions in the adrenal of dogs as the result of anoxia is significant when one recalls that many cases have been studied where death occurred in both man and animal following anoxemia and such lesions as we have observed were not present. It is suggested that the conditions of this experiment are such that one would not likely encounter its counterpart in either human cases or other experiments. To produce it, the anoxia must be of long duration—so long, in fact, that the victim dies of circulatory failure and never lives to show morphologically the anoxic insult to the adrenal cortex. This may account for the absence of reported pathologic changes even in uncomplicated acute anoxic death, such as reported by Kritzler(13). Life must persist in the dog for at least 6 hours for these lesions to develop. Animals surviving longer than 48 hours after this period of anoxia are not likely to show any more than mild cytological changes in the epithelial cells of the adrenal cortex.

[†] Supplied by Merck & Co., Rahway, N. J.

It was concluded in a previous report by one of us (H.G.S.) that an irreversible cerebral insult was produced in dogs by 6½ minutes of fulminating anoxia(9). The present study shows that this estimate is a little too short, for 4 out of 24 (17%) made apparently complete recovery from this length of exposure. At 8 minutes, however, all dogs either died or would have succumbed. These figures are very similar to those of Kabat, Dennis and Baker(14). Undoubtedly an irreversible cerebral insult takes place in both cats and dogs after approximately seven minutes of fulminating anoxia.

Summary. Dogs were given pure nitrogen to breathe and then resuscitated by lung insufflation and cardiac massage about 6½ minutes after the onset of anoxia. This was at a time when breathing and circulation had failed. These animals were sacrificed at various intervals thereafter. The earliest morphological changes that occur in the adrenal glands are pyknosis of the nucleus and coagulation of the cytoplasm of epithelial cells in the zona fasciculata and zona reticularis. Subsequently, polymorphonuclear leucocytes infiltrate these areas of the cortex. A few petechiae occur. Clinical manifestations in such anoxic dogs appear to be the result of cerebral injury and not due to adrenal insufficiency.

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Antagonism of Mercurial Poisoning in Growing Cells Investigated with Onion Root Tips. (20039)

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(Introduced by E. S. Cook.)

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Growing root tips of the bulbs of onions (*Allium cepa L.*) are highly sensitive to the organic mercurials phenylmercuric hydroxide (PMOH) and basic phenylmercuric nitrate (PMN), and the gross reactions provide delicate indicators for a range of very low concentrations(7,8,10). Aqueous solutions of alcoholic extracts of beef spleen and of yeast protected roots during short exposure to lethal concentrations of PMN(9), but did not pre-

TABLE I. Antagonism Efficiency of Cellular Extracts, Cysteine and Glutathione with PMOH Shown by Gross Reactions of Allium Roots after One Hour Exposure.

PMOH, ppm	Antagonist, %	Reaction grade in days		PMOH ppm equivalent	Relative % efficiency	Original root length, cm	MLD -100 PMOH, ppm
10	—	IV	½	—		3- 8	2.3
10	1 yeast	II	2-4	1	90	3- 7	"
10	1 "	III	3	1.5	85	6-10	"
10	1 spleen	IV	3	2.8	72	4- 7	"
10	.01 cys.	III	3	1.5	85	7-12	"
10	.5 glut.	II	4	1	90	2- 4	"
10	.5 "	III	3	1.5	85	5-10	"
10	1 yeast	II-III	2	2	80	3- 6	4
5		IV	3	—	—	5- 6	"
5	.5 "	II	3	2	60	6- 7	"
5	.5 spleen	II	5	2.5	50	5- 7	"
5	.01 glut.	II	1	.5	95	4- 6	"
	1 yeast	II	2	—		3- 6	
	.5 spleen	III	5	—		4- 6	
	.01 cys.	I	2	—		7-12	
	.01 glut.	I	4	—		6- 7	

I = Recovery, no c-tumor. II = Arrest and recovery after c-tumor. III = Permanent arrest. IV = Death. ppm = Parts per million in tap water.

vent temporary growth arrest nor the formation of terminal swellings—the colchicine-like reaction or c-tumor. It is not clear how these extracts protect the respiratory enzymes(2,3) and we have now compared the antagonism of the mercurials by the thiols cysteine hydrochloride and glutathione utilizing the gross reactions of these tissues.

Method. Bulbs of the yellow globe type of onion were germinated in the dark on glass jars over tap water. Tests were run on roots 3.5-7 cm long, as described previously(9). The test solutions were made up with the local tap water in which the roots were cultured. For convenience in tabulation and as a scale for comparison, the gross reactions have been classified in 4 grades: Grade I—slight torsion; sometimes accelerated growth rate. II—arrest and c-tumors with eventual recovery of normal growth rate. III—as II with or without recovery of slow growth in clubbed tips. IV—death, soon or later and evident first in older elongating tissue of second 5 mm of tip. Treatment for an hour in Cincinnati tap water resulted in Grade II after PMOH 0.5-1.5 ppm ($2\text{-}5.2 \times 10^{-6}$ M); III occurred in surviving organs up to the 100% lethal threshold 2.3 ppm (8×10^{-6} M = LD 100). In Palm Beach water, the reactions were induced by concentrations around twice these values(7).

Results. a. *Short treatment.* Table I shows the relative efficiency of the antagonists against PMOH 10 ppm in an hour's exposure as evaluated by the concentration of the mercurial alone which produced a similar gross reaction under the same conditions. With 1% beef spleen extract, death was only delayed and became apparent in a restricted zone as after an hour in PMOH 2.8 ppm. Table II shows the effects of different concentrations of the thiols and extracts with lethal amounts of PMOH and alone. Best protection was obtained in Palm Beach water with glutathione 0.01% at 18.8 times in excess and cysteine 47 times in excess. Glutathione 0.01% in Palm Beach also antagonized PMN 10 ppm with mixed II and III reactions. Glutathione 0.001% antagonized PMN 10 ppm (1.6×10^{-5} M) or half its molar strength with a Grade III reaction as induced by PMN 5 ppm indicating a 50% antagonism. The c-tumor effect was eliminated in short treatment only when sub-lethal (Grade III) PMOH 5 ppm was antagonized by glutathione 0.01% in bottled Deep Rock mineral spring water, and there was a reduced growth rate for a few hours. The absence of gross effects did not, however, prevent cytological abnormalities, as will be reported later.

Effective concentrations of all antagonizing agents alone in an hour's treatment produced

TABLE II. Reactions of Onion Roots to Antagonism of Lethal Doses of Organic Mercurials by Cellular Extracts and Thiols after an Hour's Exposure. Gross reactions I–IV and time in days to reach equilibrium after exposure.

				PMOH			
Antagonist	Conc., %	Antagonist alone		10 ppm, 3.4×10^{-5}M		5 ppm, 1.7×10^{-5}M	
0				IV	½ day	IV	2–3 days
AYE	1	I–II	1–2 days	II–III	2–4 days		
	.5	I–II	1 ”			II	2–3 ”
ASE	1	III	2–4 ”	IV	3 ”		
	.5	III	2–4 ”			II	5–6 ”
CSH							
8×10^{-2}M	1	$\underline{\text{IV}}$	$\underline{\text{1 hr}}$	$\underline{\text{IV}}$	$\underline{\text{¼ hr}}$		
3.2×10^{-2}M	.4	$\underline{\text{IV}}$	$\underline{\text{2 ”}}$				
3.2×10^{-3}M	.04	$\underline{\text{II}}$	$\underline{\text{3–5 days}}$				
8×10^{-4}M	.01	$\underline{\text{I}}$	$\underline{\text{2 ”}}$	$\underline{\text{III}}$	$\underline{\text{3 days}}$	II	2 ”
GSH							
1.6×10^{-2}M	.5	II	2–4 ”	II–III	3–4 ”		
3.2×10^{-4}M	.01	II	1–2 ”			II	1–2 ”

AYE—Alcoholic yeast extract; ASE—Alcoholic beef spleen extract; CSH—Cysteine Hydrochloride; GSH—Glutathione.

Underscored—Cincinnati tap water; Others—Palm Beach tap water.

some growth arrest (see Tables). Cysteine 1% with PMOH 10 ppm given for an hour caused death about as rapidly as PMOH 4.5×10^{-5} M alone due, no doubt, to the excess thiol. Concentrations of cysteine alone between 10^{-3} M and 10^{-2} M had a delayed lethal effect on the elongating tissue and above 0.25% it killed them within an hour's exposure, partly because of the low pH. Glutathione 0.5% (1.6×10^{-2} M) caused a Grade II reaction by itself.* There was a slightly greater arresting effect in the presence of PMOH (Table II) or of PMN at 10 ppm.

Considerable excess of the yeast extract was required for successful antagonism. With PMOH 10 ppm 1% of the extract gave 85-90% antagonism in Cincinnati (Table I), but with PMOH 13 ppm it failed to protect and the tissue died within 4 days. In Palm Beach PMN 20 ppm was antagonized by 1% of the extract, while 0.5% and 0.1% merely delayed the appearance of death. In Cincinnati PMN 10 ppm was antagonized by 1% and 0.8% extract to give mixed Grades II and III after 3 days, an efficiency of about 60%. Cook and Kreke(2) found that 1% yeast extract antagonized the depressant effect of PMN 10 ppm on the growth of yeast cells by 50% but had little effect in the presence of higher concentrations of the poison.

Prior immersion of the roots in the antagonists for 30 minutes failed to protect them against subsequent exposure to lethal PMOH 10 ppm.

b. *Continuous exposure.* PMN 0.2 ppm (0.32×10^{-6} M), which alone was LD 100 in 2-4 days in Cincinnati, was antagonized for 3 days continuously by cysteine 0.01% and by glutathione 0.01%. Growth was inhibited after 36 hours by the mixtures and by either thiol alone,† but all roots remained straight, rigid and normal in form throughout the treatments, and when returned to tap water, they recovered normal growth without c-tumor formation. High dilutions of various amino acids depress the growth of roots of cress seedlings(1).

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* A day after an hour in glutathione 1% and 0.5%, a girdle-like lesion, 1 mm high, of atrophied cells appeared immediately behind the meristem in both waters. The same injury occurred after an hour in PMOH 10 ppm and GSH 0.5% and some tips fractured here. Those that remained intact recovered normal growth in a few days.

† Upon return to water after 3 days in GSH 0.01%, the roots immediately attained a daily increment slightly above the controls in sturdy normal tips. Cysteine 0.01% arrested growth of immersed roots sooner. When returned to water after 3 days, they gradually recovered only from 50 to 75% of normal growth rate in the next 3 days, and the new growth was slightly tortuous. CSH 0.005% (4 x 10^{-4}M) in continuous treatment had no inhibitory effect on roots; rather it stimulated growth somewhat the first day.

Discussion. These mercurials are known to depress enzymes of the respiratory chain, partly by combination with the –SH groups, and this can be prevented by cysteine and glutathione(4). The inhibition of the cytochrome oxidase is probably not entirely due to sulfhydryl combination(6,11). It is a question, therefore, whether the successful antagonism of the depressant effects on living cells by cellular extracts is due chiefly to the presence of –SH groups in them, or whether the inactivated enzymes are side-stepped(2,3). It has been found that cysteine and glutathione in slight excess at 7×10^{-4} M will protect catalase and antagonize its depression by PMOH and PMN at 1.6×10^{-4} M(13). In the presence of air the thiols interfere with a factor which links succinic dehydrogenase and cytochrome oxidase(12). Cysteine at 13×10^{-6} M increased the inhibition of invertase by mercuric chloride 0.13×10^{-6}M(5). Macfarlane(7) suggested that growth by elongation can be recovered by onion roots when depression of the succinoxidase system has not been below 50% for short treatment as produced by concentrations of the mercurials up to approximately 10^{-6} M. The antagonists seem to reduce the effective internal concentration of the poison 50 to 95% and in some cases to an equivalent of 10^{-6} M or below. The active principle of the cellular extracts is probably a relatively small fraction of the crude extract because the latter was required in considerable excess to afford protection.

Summary. The fatal effects of PMOH and PMN at near 10^{-5} M on enlarging cells of onion root tips were successfully antagonized in an hour's exposure with cysteine and glutathione at around 10^{-4} M and by extracts of yeast (0.5-1%) and of beef spleen (0.5%). Gross reactions provided a delicate indicator for estimating the efficiency of the antagonists and showed a reduction in toxicity of 50% to 95%. Cysteine and glutathione at 0.01% also protected roots against PMN 0.2 ppm for 3 days continuously. Effective concentrations of all antagonists by themselves caused some growth arrest. The enzymic systems involved and the possible mechanism of antagonism are discussed.

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Serological Detection of Abnormal Concentrations of Serum Lipoproteins In Dogs on Cholesterol-Thiouracil Atherogenic Diets.* (20040)

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Correlation between diet or serum cholesterol concentration and the incidence of atherosclerosis in man so far has been indecisive. Rabbits and chickens can be made to develop atherosclerosis readily by increasing the cholesterol content of their diet. Atherosclerosis can be produced also in dogs with the Steiner-Kendall cholesterol-thiouracil dietary regimen. The role played by cholesterol and metabolically related unsaturated fatty acids in the production of atherosclerosis is still obscure. In a previous paper(1) dealing with these dietary factors, a successful differentiation was reported, made by means of a simple serological test, between dogs developing atherosclerosis on a Steiner-Kendall regimen and normal dogs. In that paper(1) the authors discussed the presence in the sera of dogs on a cholesterol-thiouracil atherogenic diet of an antigen which will stimulate antibody production in rabbits. This immune serum was used in precipitin tests to detect the concentration of antigen in the sera of dogs on the atherogenic diet. Using the ultracentrifuge Gofman(2) has shown abnormal serum lipoproteins and lipoprotein concentrations in the sera of rabbits and man. He has correlated these lipoproteins with the development of atherosclerosis in rabbits and man. These ultracentrifugally-defined lipoprotein molecules offered a better and more physiologic basis than total serum cholesterol concentrations on which to study the development of atherosclerosis in the dog.

Recently the presence of abnormal lipoproteins and abnormal concentrations of lipoproteins in the sera of dogs on cholesterol-thiouracil atherogenic diets has been demonstrated by means of the ultracentrifuge(3). These lipoproteins presumably contain the antigen used previously(1) to stimulate production of immune sera in rabbits. The experiments reported here were designed to test this assumption.

Procedure. Two series of dogs were placed on experimental diets and their serum lipoprotein concentrations were followed serologically and ultracentrifugally. In the first series, 4 litter mate dogs, A, B, C, and D, 9 months old, weighing from 12 to 16 kg, received daily diets as follows in addition to their stock diets: Dog A—cholesterol (Eastman—30 g) and thiouracil (Lilly—1.2 g); Dog B—cottonseed oil (Procter & Gamble—60 ml) and thiouracil (1.2 g); Dog C—thiouracil (1.2 g); and Dog D—stock control diet only. After nineteen weeks their individual sera were fractionated and analyzed for S_f 2-30 lipoproteins in the following manner: Ten ml of serum were adjusted to a density of 1.062 ± 0.001 by the addition of 22% sodium chloride solution. Twelve ml of this serum preparation were placed in a Spinco Model L preparative ultracentrifuge for 10 hours at 40,000 rpm (105,000 x g) at a temperature of 15° ± 1°C. The top 1.2 ml of this preparation were then withdrawn by means of a curved capillary tube attached to a 2 ml glass hypodermic syringe. This fraction represented a lipoprotein concentration 6 times that of the unfractionated serum. Of this supernatant fraction, 0.8 ml was placed in the analytical Spinco Model E ultracentrifuge at 59,780 rpm (260,000 x g) for 30 minutes at a temperature of 27°C ± 1°C. Photographs were taken at intervals while the centrifuge was at speed. From these photographs lipoprotein peaks and the lipoprotein concentration of the fraction were determined.

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* This work was supported by a grant from the Central Ohio Heart Association. The authors acknowledge with thanks the help of Dr. Quentin Van Winkle and Mr. Richard H. Leet of the Department of Chemistry in consultation and experimental management of the ultracentrifugal work included. The authors also acknowledge the kindness of Dr. Kenneth Kohlstaedt of the Eli Lilly Co. for supplying thiouracil for this investigation.

In the fraction from the serum of Dog A, 3 peaks (S_f 2-7, S_f 8-11 and S_f 13-17) were observed; sera of normal dogs contained only one peak (S_f 2-7). From the 1.2 ml supernatant obtained from the serum of Dog A on the cholesterol-thiouracil atherogenic diet, two 0.5 ml aliquots were taken. Each was made up to a volume of 5 ml by addition of sterile 0.9% sodium chloride solution. These preparations were then injected intravenously into two young adult white rabbits, a_1 and a_2, at 7 day intervals for a total of 4 injections. Seven days after the last injection, these rabbits were bled aseptically from the heart. The sera obained from these rabbits were used in precipitin tests against the unfractionated sera from Dogs A, B, C, and D.

These *precipitin tests* were performed as follows: with unfractionated dog serum diluted 1:10 in 0.9% sodium chloride solution, doubling dilutions were made to 1:640. Four-tenths ml of each dilution was placed in precipitin tubes and to each tube was added 0.05 ml of immune serum from rabbit a_1. Similar titrations were performed using the immune serum from rabbit a_2. Appropriate controls were included with each titration. Immediately after adding the immune rabbit serum, the precipitin tubes were incubated in a water bath at a temperature of 37° ± 1°C for one hour. The tests were placed in a refrigerator at 4°C and the titers were read the next day. A second series of 2 dogs, S and R, 2 to 3 years old, weighing 12 to 14 kg, were placed on daily diets of cholesterol (30 g) and thiouracil (1.2 g), and a stock control diet, respectively. After these dogs had been on their diets for 8 weeks, their unfractionated sera also were titered against immune rabbit sera a_1 and a_2. Precipitin tests were performed weekly for periods of 6 and 7 weeks on the first and second series of dogs, respectively. Random samples of serum from 2 other dogs on the stock diet were similarly examined.

The *unfractionated sera* from Dogs A, B, C, D, S, and R also were titered against sera from rabbits injected with unfractionated sera from Dogs A, B, C, and D respectively. Other controls included precipitin tests with sera of normal rabbits against the unfractionated sera of Dogs A, B, C, D, S, and R. Both normal rabbit sera and a_1 and a_2 immune rabbit sera also were tested against a saturated solution of cholesterol in 0.9% sodium chloride solution, and cholesterol added in excess to normal dog serum.

Total serum cholesterol was determined by the Sperry-Schoenheimer method in Dogs A, B, C, and D at intervals while they were on the experimental diets. The serum of a 77-year-old man with diabetes mellitus and arteriosclerotic heart disease, as well as the sera of 3 other men, aged 26, 27, and 53, in whom no vascular disease had been detected, were tested by precipitin technic against immune rabbit sera a_1 and a_2. *Nitrogen* was determined (micro-Kjeldahl) in order to standardize the ultracentrifugal lipoprotein fraction used as antigen. After determining optimal proportions and equivalence zones by precipitin tests using the sera of Dogs S and R against the immune rabbit sera a_1 and a_2, nitrogen determinations were made on the precipitates.

Results. 1) The concentrations of S_f 2-30 lipoproteins in Dogs A and S on the cholesterol-thiouracil atherogenic regimen were consistently elevated above the concentrations found for Dogs B, C, D, and R; the precipitin titers were of the order of 1:100 or greater in Dogs A and S, usually ranging in the vicinity of 1:500. These titers occurred in the samples shown in Table I as having an S_f 2-30 lipoprotein level varying between 112 and 176 mg %. These lipoproteins are represented by 3 peaks, S_f 2-7, found in all dogs studied, and S_f 8-11 and S_f 13-17, found only in dogs on atherogenic dietary regimens.

2) The concentrations of S_f 2-30 lipoproteins in dogs on non-atherogenic diets: Dog B (on cottonseed oil and thiouracil), Dog C (on thiouracil), and Dogs D and R (on the stock control diet), were consistently low; the precipitin titers were 1:100 or less. These titers occurred with S_f 2-30 lipoprotein levels varying between 8 and 51 mg % as shown in Table I. Similar low titers were found when random samples of sera from two other normal dogs on stock diets were tested. This lipoprotein is represented only by the S_f 2-7 peak found in all dogs studied.

TABLE I. Comparison of Lipoprotein Precipitin Titers and S_f 2-30 Lipoprotein Concentrations in Dog Sera.

Date samples drawn	Titer/lipo.											
	Dog A		Dog B		Dog C		Dog D		Dog R		Dog S	
7/14	1:500		1:10		1:10		1:100		1:10	8	1:500	112
14	1: 80		1:20		1:20		1: 80		1:40		1:640	
21	1:100	172	1:10		1:10		1: 10	17	1:10	11	1:500	123
28	1:500	171					1:100		1:10		1:500	126
8/ 4	1:320	125		20		29		10	1:40		1:400	
11	1:160		1:20		1:40		1: 40		1:20	10	1:640	142
11	1:320		1:40		1:40		1: 80		1:40		1:640	
11	1:640 A_f		1:80 B_f		1:80 C_f		1: 80 D_f		1:80 R_f		1:640 S_f	
18	1:640	145	1:40	36	1:80	32	1: 80	11	1:20		1:640	
18	1:640 A_f		1:80 B_f		1:80 C_f		1: 80 D_f					
21								10				
25			1:80	31	1:80	51			1:40	11	1:320	176
27						44						

Dog A—Cholesterol-thiouracil + stock diet. Dog B—Cottonseed oil-thiouracil + stock diet. Dog C—Thiouracil + stock diet. Dog D—Stock diet. Dog R—Stock diet. Dog S—Cholesterol-thiouracil + stock diet.

A_f, B_f, C_f, D_f, R_f, and S_f are the ultracentrifugal lipoprotein fractions S_f 2-30 from the respective dog sera used as antigen in precipitin tests.

Lipoprotein concentration expressed in mg %.

3) *Precipitin tests* using the unfractionated sera of Dogs A, B, C, D, S, and R against sera from rabbits previously injected with unfractionated sera from Dogs A, B, C, and D, respectively, showed no significant differences in titer.

4) Sera of normal rabbits, when tested against the unfractionated sera of Dogs A, B, C, D, S, and R produced no precipitate, even with dilutions of dog serum as low as 1:5.

5) Neither normal rabbit sera, nor a_1 and a_2 immune rabbit sera, when tested against a saturated solution of cholesterol in 0.9% sodium chloride solution, or against cholesterol added in excess to normal dog serum produced any precipitate, even with dilutions of cholesterol solution as low as 1:10.

6) Total serum cholesterol concentrations, determined at intervals by the Sperry-Schoenheimer method in Dogs A, B, C, and D while on the experimental diet, were not related to precipitin titers or to lipoprotein concentrations.

7) Immune rabbit sera a_1 and a_2, when tested against the serum of a 77-year-old man with diabetes mellitus and arteriosclerotic heart disease, gave precipitin titers of 1:500, whereas the sera of 3 other men, 26, 27, and 53 years old, respectively, when tested in the same manner, produced no reactions, even with a dilution of 1:10.

8) Nitrogen determinations demonstrated a concentration of antigenic protein nitrogen in the serum of Dog S of 3750 μg of nitrogen per ml which was 7 times the concentration found in the serum of Dog R (550 micrograms of nitrogen per ml).

Discussion. The results presented here confirm and extend those previously reported (1) and describe a simple serological method for differentiating dogs on cholesterol-thiouracil atherogenic diets from other dogs by detecting their abnormally increased serum lipoprotein concentrations. Studies now in progress have given additional confirmation to the work discussed here. Since the rabbits were specifically immunized against the ultracentrifugally prepared fraction of dog serum which was photographically defined as containing a high concentration of lipoproteins S_f 2-30; it is presumed that the immune sera produced in these rabbits were a response to the antigenic properties of these lipoproteins. No better way of purifying and isolating these lipoprotein fractions from dog serum has yet been described.

No attempt has been made in this paper to quantitate the precipitin titers obtained with either total or abnormal lipoprotein concentration, since finite quantities of albumin in the S_f 2-30 fraction used as antigen may influence the titer to some degree. Further studies are now in progress to quantitate this serological test.

Summary. By immunizing rabbits with a lipoprotein fraction obtained from the serum of a dog on a cholesterol-thiouracil atherogenic diet, it has been possible to produce immune sera which will detect the abnormally increased concentration of serum lipoproteins in dogs on cholesterol-thiouracil atherogenic diets and which will react with the serum from an atherosclerotic man. Sera of normal dogs tested with this immune serum by the precipitin technic, consistently show a lipoprotein concentration significantly less than that observed in the case of dogs on the atherogenic dietary regimen. Sera from three men in whom no vascular disease had been detected, did not react with the immune rabbit sera.

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In vitro Studies with 1-hydroxy-2(1H)pyridinethione. (20041)

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Shaw *et al.*(1) in their work on analogs of aspergillic acid reported the preparation and properties of 1-hydroxy-2(1H)pyridinethione, the sodium salt of which is designated MC 3277.* Because of its striking activity against a very wide group of microorganisms further studies were undertaken. The present report will discuss certain antibacterial and antifungal characteristics of this compound.

Assay of MC 3277. A turbidimetric tube assay has been developed using as an assay organism, *Saccharomyces cerevisiae* Squibb strain, and Difco penassay broth modified to contain 1% dextrose concentration as a test medium. Otherwise the assay procedure of Donovick *et al.*(3) is used unchanged. Statistical analysis† of data obtained with the assay shows that for a single assay on any one day, the assay will be within 8.5% of the true value 95% of the time.

In vitro spectrum. A. *Antibacterial.* The sensitivities of a group of microorganisms to MC 3277 were determined as follows: A 2-fold dilution procedure was used with incubation for 17 hours at 37°C for most organisms. Unless otherwise stated, Difco penassay broth was used as a test medium. Cultures in the logarithmic phase of growth with approximately 1,000 organisms per ml were used where possible. The amount of MC 3277 required to inhibit completely the growth of the organism as measured by an absence of turbidity is reported as the Minimal Inhibiting Concentration (M.I.C.). In Table I are listed the M.I.C.'s for a representative group of microorganisms. The M.I.C.'s range from 0.006 μg/ml for *M. tuberculosis* var. *bovis* Strain BCG to 4.0 μg/ml for *Pseudomonas aeruginosa*. The gram-positive organisms are most sensitive but the range of activity for the gram-negative group, from 1.5 μg/ml for *Klebsiella pneumoniae* and *Salmonella typhosa* to 4.0 μg/ml for *Ps. aeruginosa* is of a high order.

B. *Antifungal.* The action of MC 3277 against a group of molds, yeasts and yeast-like fungi was determined by the streak-plate method using a malt-yeast extracts dextrose agar. In Table II are reported the M.I.C.'s found for these fungi. The activity

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† The authors are indebted to Mrs. Mavis Carroll of the Squibb Institute for Medical Research for the statistical study.

TABLE I. Antibacterial Spectrum of MC 3277.

Organism	M.I.C. in µg/ml	Medium
Micrococcus pyogenes var. *aureus* 209P	.13	Difco penassay broth
Klebsiella pneumoniae	1.5	”
Pseudomonas aeruginosa	4	”
Streptococcus faecalis	.5	”
Shigella dysenteriae	.5	”
Streptococcus pyogenes C-203	.25	Difco brain heart infusion
Salmonella schottmülleri	2.5	Difco penassay broth
Aerobacter aerogenes	3.5	”
Escherichia coli	2.5	”
Proteus vulgaris	.7	”
Mycobacterium tuberculosis var. *bovis* strain BCG	.006	Modified Kirchner's*
Salmonella typhosa	1.5	Difco penassay broth
Lactobacillus acidophilus	.4	”
Clostridium septicum	.4	Fluid thioglycollate medium
Brucella abortus	.4	Tryptose semi-solid
Neisseria catarrhalis	.6	Difco brain heart infusion
Corynebacterium pyogenes	.3	Squibb diphtheria medium
Streptococcus agalactiae	.08	Difco penassay broth
Bacillus subtilis	.06	”

* Donovick, R., Pansy, F., Stryker, G., and Bernstein, J., *J. Bact.*, 1950, v59, 667.

TABLE II. Antifungal Spectrum of MC 3277.

		M.I.C. in µg/ml of agar
Aspergillus niger		.6
A. fumigatus		.3
Penicillium notatum		.3
Ceratostomella ulmi		.08
Trichophyton mentagrophytes #1920		1
T. mentagrophytes Squibb		.15
T. tonsurans		1
T. rubrum		1
T. schoenleini		.5
T. violaceum		2
T. faviforme var. *discoides*		.03
T. faviforme var. *ochraceum*		.03
Microsporum audouini		.3
M. audouini (Kligman strain)		<.06
M. canis		1
Epidermophyton flocossum		.25
Sacch. cerevisiae		.08
S. pastorianus		.04
Candida albicans		.08
Cryptococcus neoformans	(in Difco Penassay broth + .5% glucose)	.10

of the pyridinethiones against the fungi is particularly striking. The most resistant organism found was *Trichophyton violaceum* which was inhibited by 2 µg/ml. Most of the other fungi are much more sensitive. Of particular interest are the very high activities against *Candida albicans* and *Cryptococcus neoformans.*

Action of various body fluids on the action of MC 3277. Since, in preliminary tests vs. *Streptococcus pyogenes* C-203, MC 3277 showed no activity in mice, it was of interest to determine whether this compound was easily inactivated by body fluids. The action of saliva, cerebrospinal fluid and serum on MC 3277 was accordingly determined. More extensive inactivation studies are reported by Thomas *et al.*(2).

A. Saliva. Four organisms, *M. pyogenes,* var. *aureus, Streptococcus pyogenes, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa,* were tested in a basal medium (Difco penassay broth) and in the same medium with 30% of the water substituted by saliva. The media were sterilized by Seitz filtration and M.I.C.'s of 1-hydroxy-2(1H)pyridinethione were determined by a 2-fold procedure in the two media. The results, reported in Table III, show some degree of inactivation for *Strep. pyogenes, K. pneumoniae,* and *Ps. aeruginosa,* and a high degree of inactivation for *M. pyogenes* var. *aureus.* In a similar manner MC 3277 was tested against *Saccharomyces cerevisiae* in the presence and absence of 0.1% mucin. An inactivation of MC 3277 of about 25% was observed.

B. *Cerebrospinal fluid.* Media were prepared as in A above using 30% human cerebrospinal fluid (CSF) as the water substitute. The M.I.C.'s of MC 3277 were obtained against *M. pyogenes* var. *aureus* and *Crypto-*

TABLE III. Action of Saliva on Activity of 1-hydroxy-2(1H)pyridinethione.

Organism	M.I.C. in μg/ml: In basal medium	M.I.C. in μg/ml: Basal medium + 30% saliva
M. pyogenes var. *aureus* 209P	.15	3.5
Strep. pyogenes C-203	.3	.6
K. pneumoniae	2	3
Ps. aeruginosa	5	6

TABLE IV.
Effect of Serum on Activity of Several Concentrations of MC 3277 against *Saccharomyces cerevisiae.*

Concentration, μg/ml	M.I.C. in μg/ml at 0 hr	1 hr	3 hr	24 hr
A) 50% Bacto beef serum at 37°C				
1000	.0261	.0230	.0245	.0285
500	.0277	.0238	.0238	.0245
100	.0230	.0223	.0277	.0277
5	.0328	.0384	.0396	.0592
B) Distilled water at 37°C				
1000	.0277	.0238	.0253	.0245
500	.0253	.0260	.0268	.0317
100	.0245	.0238	.0245	.0223
5	.0306	.0317	.0357	.0317

coccus neoformans in both media. The M.I.C. of 0.15 μg/ml for *M. pyogenes* var. *aureus* and 0.10 μg/ml for *C. neoformans* was unchanged by the presence of CSF after 2 days incubation at 37°C.

C. *Serum.* Weighings of MC 3277 were made up in 50% sterile Bacto beef serum and sterile distilled water. The levels made up were 1,000 μg/ml, 500 μg/ml, 100 μg/ml, and 5 μg/ml. Aliquot portions were drawn off and assayed immediately and the samples then placed at 37°C. At one hour, 3 hours, and 24 hours, portions were drawn off and assayed by the method described above. The data (Table IV) indicate that at concentrations of 1,000 μg/ml, 500 μg/ml, and 100 μg/ml, there is no apparent inactivation. At 5 μg/ml in 50% Bacto beef serum, however, there appears to be some inactivation after 24 hours.

Summary and conclusions. An antibacterial and antifungal spectrum for the sodium salt of 1-hydroxy-2(1H)pyridinethione as well as an assay method for the same is presented. *In vitro* the substance is partially inactivated by saliva and mucin, by serum at low concentration, but not by human cerebrospinal fluid. The inactivation by saliva, mucin, and serum however may be of relatively minor importance in view of the extraordinarily high activity displayed by this compound.

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Antibiotic Combinations and Resistance to Antibiotics: Penicillin-Erythromycin and Streptomycin-Erythromycin Combinations *in vitro*.* (20042)

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The emergence of penicillin-resistant strains of staphylococci during prolonged exposure to that antibiotic *in vitro* or in the course of therapy is now a well established phenomenon. In many large hospitals where penicillin is used very extensively this phenomenon is probably responsible for the complete reversal from predominantly sensitive to predominantly resistant strains of staphylococci among those isolated from infected materials and carriers in the hospital populations(1-6). It has recently been demonstrated that the new antibiotic erythromycin is highly and equally active against both penicillin-sensitive

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† With technical assistance of Clare Wilcox and Marilyn K. Broderick.

and penicillin-resistant staphylococci(7-10). It was also shown that staphylococci and other erythromycin-sensitive organisms may rapidly acquire high degrees of resistance to erythromycin during repeated subcultures in the presence of that agent(11). Several cases have been reported in which there was a change from sensitive to resistant strains of staphylococci and streptococci isolated from blood cultures during the course of treatment with erythromycin(8,9). Previous studies have also shown that repeated subcultures of various bacteria in the presence of streptomycin results in rapid development of high degrees of streptomycin-resistance in strains originally sensitive to that antibiotic(12,13) and clinical counterparts of this phenomenon have been noted in the rapid emergence of highly resistant strains during the course of treatment with streptomycin(14). The present study was undertaken to determine whether the development of resistance of staphylococci to penicillin and erythromycin *in vitro* could be suppressed by exposure of sensitive strains to both agents simultaneously. Similar studies were also made to determine whether the development of resistance of staphylococci and of enterococci to streptomycin and erythromycin could be prevented or suppressed *in vitro* by the use of these 2 antibiotics in combination.

Materials and methods. 6 coagulase-positive strains and 1 coagulase negative strain (No. 1) of *Staphylococcus aureus*, all of them hemolytic and each sensitive to both penicillin and erythromycin, were chosen for the first part of this study; their sources are listed in the upper part of Table I. These strains had each been obtained from single colony cultures, stored at –25°C and subcultured in broth prior to use in this study. Crystalline potassium penicillin G was obtained commercially; the erythromycin (Ilotycin, Lilly) was furnished in a purified crystalline form by Dr. John W. Smith of Lilly Research Labs. and contained 890 μg per mg. A stock solution of erythromycin was prepared and kept frozen at –25°C when not in use. Stock solutions of penicillin were freshly prepared each week and stored at 5°C when not in use. The method used for increasing resistance to antibiotics was similar to that previously employed in this laboratory(11,13,15); in this part of the study subcultures were made daily on graded concentrations of antibiotics incorporated in blood agar and the inoculum each time consisted of a broth suspension made from the surface growth of the plate containing the highest concentration of antibiotic(s) on which the growth was maximal or nearly so. When the 2 antibiotics were used together, a constant ratio of 16 times as much erythromycin as penicillin was used, this ratio having proved optimum in preliminary tests. The strains used in the studies with streptomycin and erythromycin were all recently isolated from patients with severe illness; their sources are listed in the lower portion of Table I. None of the patients had previously received treatment with either streptomycin or erythromycin. The organisms had undergone very few subcultures, most of them only 1 or 2, prior to the isolation of the single colony cultures with which these studies were made. In each instance the original colony chosen was 1 of 10 picked from a pour plate of a fully grown culture so diluted as to yield widely separated and discrete colonies; each of the 10 colonies was tested for sensitivity to the antibiotics under study and all were found to be identical within the limits of the 2-fold dilution method. In this part of the study the inoculum for agar plates was obtained by subculturing to antibiotic-free broth

TABLE I. Sources of Strains Used.

Strain		Source	Diagnosis
Penicillin-erythromycin series			
Staphylococcus	1	Pus	Abscess
	2	Throat	Pneumonia
	3	Pus	Furuncle
	4	Urine	Pyelonephritis
	5	Sputum	Pneumonia
	6	Pus	Paronychia
	7	Sinus	Osteomyelitis
Streptomycin-erythromycin series			
Staphylococcus	H	Blood	Endocarditis
	S	Sinus	Osteomyelitis
	W	Blood	Peritonitis
	M	Urine	Pyelonephritis
	U	Blood	Septicopyemia
	A	Urine	Pyelonephritis
	2	Sinus	Osteomyelitis
	197	Sputum	Pneumonia
Enterococcus	2	Urine	Pyelonephritis

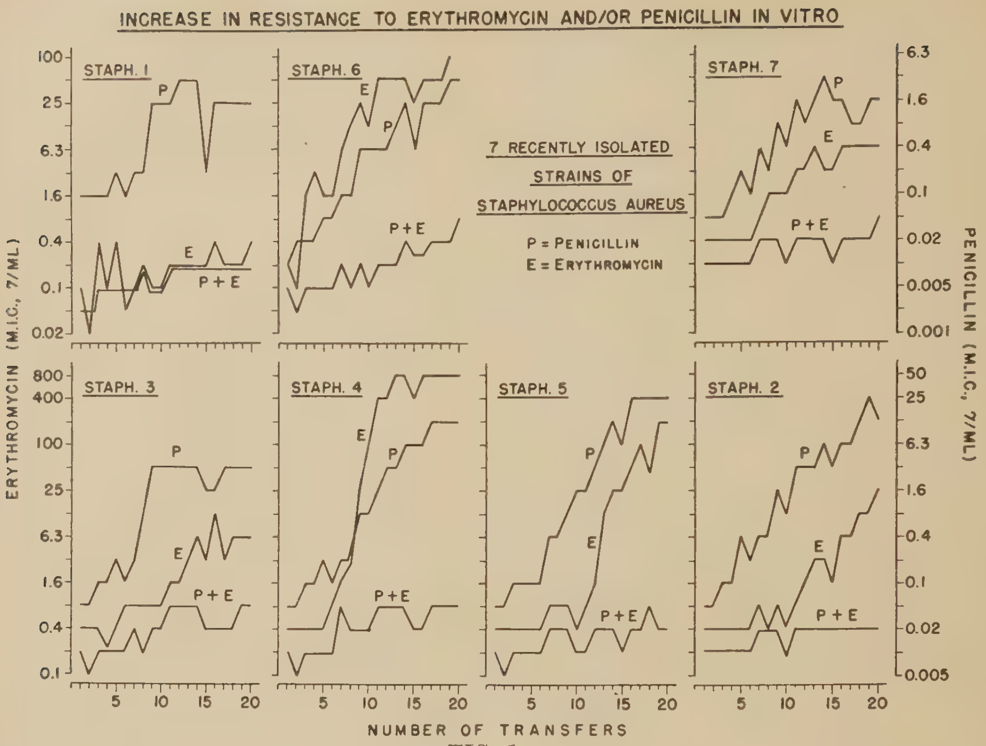

FIG. 1.

from the plate containing the maximum concentration of antibiotic which permitted good growth at 18 hours; the broth was allowed to incubate for 6 hours and the resulting growth used for the next transfer on antibiotic agar. Three parallel series of transfers were carried out in each instance:—one in erythromycin, a second in streptomycin and a third with both antibiotics combined. For the third series, carried out with staphylococcal strains H, 2 and 197 and Enterococcus 2, a constant ratio of 16 parts of streptomycin to 1 of erythromycin was used throughout and for the remaining strains this ratio was 256 to 1; these ratios were found to be optimal in preliminary tests with the parent strains. In part of this study the serial daily transfers were made in increasing concentrations of antibiotics incorporated in broth, each transfer being essentially a test for sensitivity done with serial 2-fold dilutions in broth. Streptomycin sulfate was used and was obtained commercially. Throughout these studies the sensitivity of a strain is defined as the minimum concentration of antibiotic producing complete inhibition of growth in 24 hours; it is expressed in μg/ml and 1 unit of penicillin was taken as equal to 0.6 μg. Coagulase tests were carried out daily or on alternate days with all of the strains of staphylococci in each series.

Results with penicillin and erythromycin. The changes in resistance of the 7 strains during the daily subcultures on agar containing penicillin (P), erythromycin (E), or both (P + E) are shown graphically in Fig. 1. In each instance there was a more or less steady increase in resistance to penicillin and, except in the case of *Staph. 1,* similar increases in resistance occurred during repeated subcultures on erythromycin. Considering the crudeness of the method, the rates of increase in resistance of each strain to the individual agents were remarkably similar. When the subcultures were made on agar containing both antibiotics, only minor increases or none at all developed in the resistance of the staphylococci to the combination of penicillin and erythromycin.

The changes in resistance to the 2 antibiotics, individually and together, that resulted

TABLE II. Sensitivity of 7 Strains of *Staphylococcus aureus* to Penicillin and Erythromycin before and after 20 Subcultures on Agar Containing One or Both of These Antibiotics.

Strain No.	Minimum complete inhibiting concentration, μg/ml									
	Penicillin				Erythromycin				Penicillin + erythromycin	
	0*	P—R	E—R	P+E—R	0	P—R	E—R	P+E—R	0	P+E—R
1	.1	1.6	.1	.1	.4	.4	.4	.2	.002+ .05	.01 + .2
2	”	12.5	”	.05	”	”	25	.8	.01 + .2	.02 + .4
3	”	3.1	”	.2	”	”	6.3	3.1	”	.05 + .8
4	”	12.5	.05	.05	”	”	800	”	”	”
5	”	25	.1	.1	”	”	200	1.6	”	.02 + .4
6	”	3.1	”	.05	”	”	100	>800	”	.1 +1.6
7	”	1.6	”	”	”	”	6.3	.8	”	.05 + .8

* 0 = Parent strain (not previously exposed to antibiotics); P—R = after 20 transfers on penicillin; E—R = after 20 transfers on erythromycin; P+E—R = after 20 transfers on agar containing both penicillin and erythromycin (the first value is for penicillin, the second is for erythromycin).

from the 3 series of transfers are listed in Table II. Increases in resistance to the homologous antibiotics ranged from 16-fold to 256-fold for penicillin alone and (excluding Strain No. 1) from 16-fold to more than 1028-fold for erythromycin alone, as compared with only 2- or 4-fold increases in resistance to the 2 agents in combination (except for Strain No. 6 which increased 8-fold). Cross-resistance between the individual antibiotics did not develop. Moreover, after 20 subcultures on agar containing both penicillin and erythromycin all 7 strains retained their original sensitivity to penicillin and 3 of the strains also retained their sensitivity to erythromycin; the resistance to the latter, however, increased 4-fold or 8-fold in 3 strains and more than 2048-fold in Strain No. 6.

Coagulase production. Strain No. 1 failed to produce coagulase before and at the end of the 3 series of exposures to the antibiotics. The remaining strains all produced coagulase and this property remained unaltered after the transfers with the antibiotics singly or in combination, with 2 exceptions:—after the 20 transfers on penicillin agar, strains 3 and 7 failed to produce coagulase.

Results with streptomycin and erythromycin. Agar plate method. The changes in sensitivity to the homologous antibiotics that occurred during serial transfers on agar containing streptomycin, erythromycin or both were studied with 2 strains of staphylococci (No. 2 and 197) and with Enterococcus 2. The results obtained with the latter are shown in Fig. 2 and those obtained with the staphylococci were very similar. As in other studies employing this crude method, there are irregularities but, in general, each of the strains, after a variable lag, showed a marked increase in resistance to erythromycin and to streptomycin during the successive transfers in the corresponding antibiotic. Resistance to the combined antibiotics developed later and to a lower degree in the staphylococci and was apparently not sustained in the case of the Enterococcus. In the case of all of the organisms used in this study the sensitivity of the original strains to the combination of erythromycin and streptomycin was somewhat more

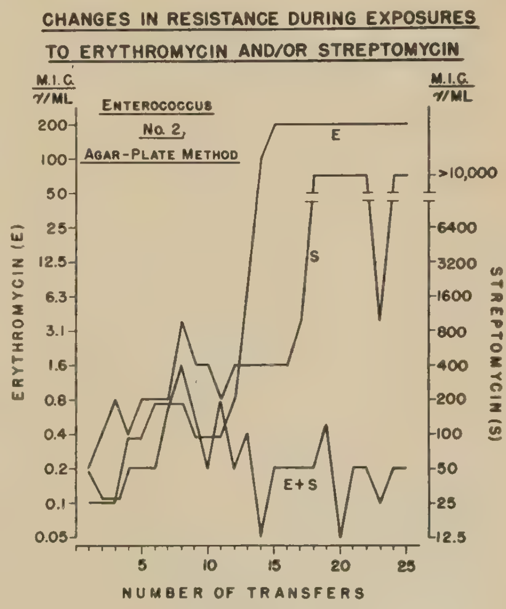

FIG. 2.

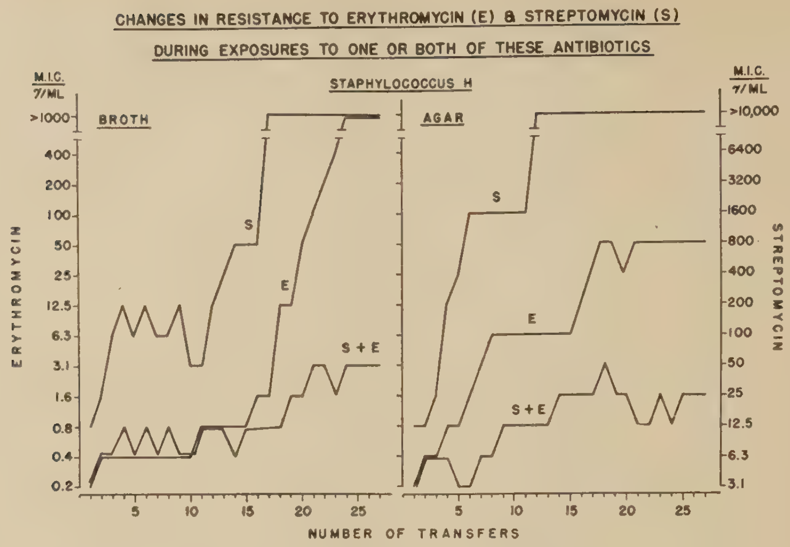

FIG. 3.

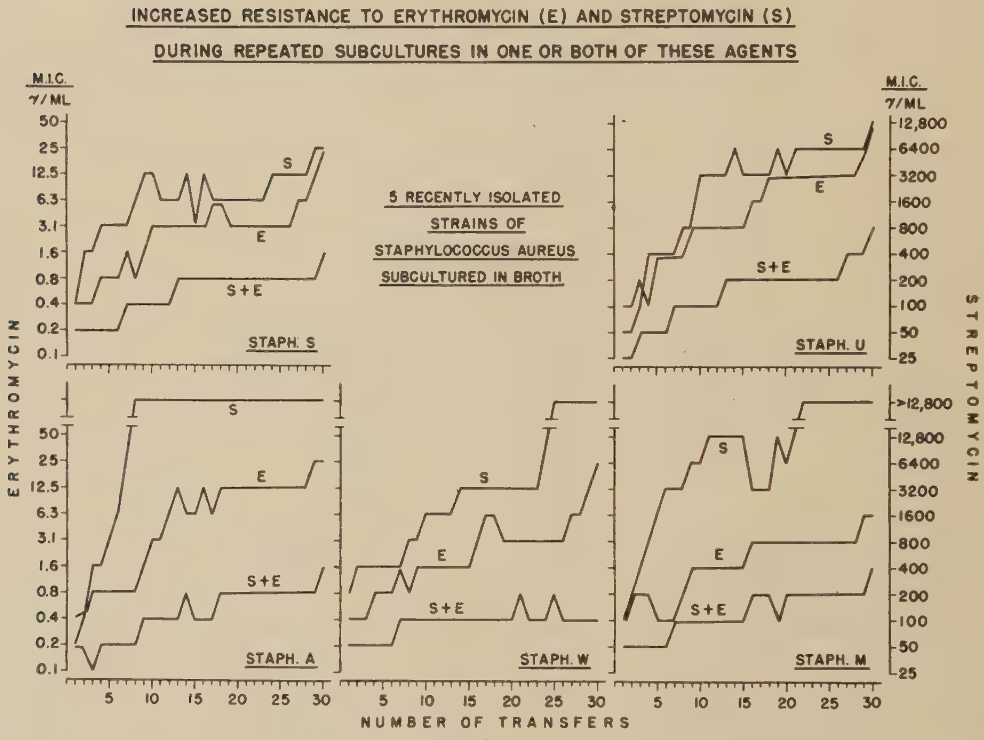

FIG. 4.

than additive.

Comparison of the plate and broth methods. Because transfers in broth could be made somewhat more quantitatively than in the agar method as here employed, more uniform changes in resistance might be anticipated if the broth method were used. *Staph. H* was, therefore, studied with parallel transfers in broth and agar; the results are shown in Fig. 3. Resistance appeared to develop somewhat

TABLE III. Sensitivity to Erythromycin and Streptomycin before and after Repeated Subcultures in the Presence of One or Both of These Antibiotics.

	Minimum complete inhibiting concentration, μg/ml									
	Erythromycin (E)				Streptomycin (S)				Erythromycin + streptomycin	
Strain	0	E—R	S—R	E+S—R	0	E—R	S—R (×1000)	E+S—R	0	E+S—R
Strains transferred in broth										
Staph. H	.4	>1000	.8	3.1	12.5	12.5	>10	50	.2 + 3.1	1.6 + 25
S	"	25	.4	"	100	100	.64	400	.2 +50	3.1 +800
W	"	12.5	.2	.8	200	50	>12.8	"	"	.4 +100
M	"	6.3	.4	6.3	100	100	"	1600	"	1.6 +400
U	"	50	.2	3.1	50	25	12.8	800	.1 +25	3.1 +800
A	"	25	.4	1.6	50	50	>12.8	"	.2 +50	1.6 +400
Strains transferred on blood-agar										
H	.2	50	.2	3.1	12.5	50	>10	50	.2 + 3.1	1.6 + 25
2	.4	12.5	.2	.2	6.3	25	"	25	"	"
197	"	200	.2	1.6	25	12.5	"	"	.4 + 6.3	"
Entero. 2	.1	"	.04	.2	50	100	.64	100	.1 + 1.6	.2 + 50

0 = Parent strain (not previously exposed to antibiotics); E—R = after repeated subcultures with erythromycin; S—R = after repeated subcultures with streptomycin; E+S—R = after subcultures with both erythromycin and streptomycin; Staph. H was transferred 36 times in antibiotic broth and 27 times in antibiotic agar; the other strains were transferred 30 times in antibiotic broth and 20 times on antibiotic agar.

more uniformly and a higher degree of resistance to erythromycin was attained when the broth method was used. Qualitatively, however, the results with the 2 methods were similar.

Broth-dilution method. An additional group of 5 strains of staphylococci were each carried through the 3 series of 30 transfers in broth; the results are shown in Fig. 4. In each instance, resistance to erythromycin increased more slowly, and in some of the strains the total change in resistance achieved (in terms of "fold increase") was less than with streptomycin. For each of the 5 strains, however, the resistance resulting from the transfers in the combination of erythromycin and streptomycin was slower in developing and was of a significantly lower order of magnitude than that which resulted from exposures to each antibiotic separately.

Cross-resistance. At the completion of the study all of the strains resulting from the various transfers were tested for sensitivity to streptomycin and erythromycin by the plate-dilution method. The results are shown in Table III. The parent strains in this study had been maintained in the frozen state at –25°C during the course of the study and were subcultured and tested at the same time for comparison. It is seen that, within the limits of the method, no cross resistance to erythromycin or streptomycin resulted from the subcultures in the presence of these agents individually. The organisms which had been transferred in the combination of the 2 antibiotics did show increases in resistance when tested with each antibiotic separately; these increases in resistance were, in general, of the same order of magnitude as the increases in resistance that occurred to the 2 agents used in combinaton. Additional tests for *sensitivity* to penicillin, bacitracin, aureomycin, terramycin, chloramphenicol, polymyxin B (Aerosporin) and neomycin were carried out with staphylococcal strains W, S, M, U and A, using the parent cultures and the final transfers of the 3 antibiotic series in each instance. The parent strains were each resistant to > 400 μg/ml of penicillin and to >200 μg/ml of polymyxin and were inhibited by 6.3 μg/ml of bacitracin; no changes in resistance were noted with respect to these 3 antibiotics in any of the 5 strains. Four of the 5 strains showed no change in resistance to aureomycin and terramycin (both grew in >400 μg/ml of each agent) as a result of the exposures to erythromycin and/or streptomycin; *Staph. M,* however, was initially resistant to >400 μg/ml of both aureomycin and terramycin but the erythromycin-resistant variant of this organism was completely inhibited by 25 μg and 6.3 μg/ml of these agents, respectively. All 5 staphylococcal strains increased in resistance to neomycin as a result of the repeated exposures to streptomycin used alone and in combination with erythromycin, but they retained their original sensitivity to neomycin after they had become resistant to erythromycin. The cross-resistance between streptomycin and neomycin was similar to that previously observed in this laboratory(15,16).

Biological reactions. The following observations were made simultaneously on the parent strains and on the final transfers of each of the 3 series of subcultures of the 6 staphylococcal strains done in antibiotic-containing broth: 1) hemolysis on blood agar, 2) pigment production, 3) coagulase reaction, 4) coagulation of milk and 5) fermentation of lactose, mannitol and sucrose. All of these organisms remained hemolytic and coagulase positive and they continued to produce the characteristic yellow pigment but some of the strains which had become resistant to either erythromycin or streptomycin apparently produced the pigment either in smaller amounts or more slowly, and some of them grew less luxuriantly than their parent strains on the surface of blood agar. The fermentation reactions were the same in the parent and derived strains in every instance.

Discussion. These results indicate clearly that the strains of staphylococci used in the first part of this study developed resistance to penicillin and erythromycin rapidly and to significant and high degrees during repeated subcultures in the presence of increasing concentrations of the homologous agent. On the original strains, the combination of these 2 antibiotics had an effect which was somewhat greater than additive. The 20 subcultures on penicillin together with erythromycin left the strains essentially unchanged with respect to their sensitivity to penicillin alone and increased only slightly their resistance to erythromycin alone and to the 2 antibiotics in combination. The reason for the aberrant result in the case of strain No. 6 is not clear at this time and requires further investigation. It was not associated with penicillinase production by this strain after exposure to the 2 agents. Moreover, the reason for the difference of the effects of exposure to erythromycin on coagulase production in this study as compared with previous results(11) is not clear.

The results obtained with the combination of streptomycin and erythromycin were very similar; in the presence of each antibiotic individually, resistance developed rapidly and to a high degree but when both were used together in a fixed and predetermined proportion of each, resistance to the combination of these agents was delayed and depressed. Moreover, following the repeated subcultures in increasing concentrations of the combination of these 2 antibiotics, the resistance to the individual agents increased only to about the same degree as to the combination. There was no evidence, however, that the presence of either of the antibiotics in the 2 combinations studied here reduced in any way the effectiveness of the other agents.

Summary and conclusions. 1. During 20 subcultures on blood agar containing increasing concentrations of penicillin or erythromycin each of 7 strains of *Staphylococcus aureus* increased markedly in resistance to the homologous antibiotic without affecting its sensitivity to the other agent. The combination of penicillin and erythromycin exerted slightly more than an additive effect on the parent strains. During 20 parallel subcultures on agar containing both antibiotics, the organisms retained their original sensitivity to penicillin alone but most of them increased slightly in resistance to the 2 agents in combination and to erythromycin alone, while one strain increased markedly in resistance to the latter agent. Coagulase production by these staphylococci remained unaltered following the exposures to these antibiotics except for 2 strains which lost this property after they became resistant to penicillin. 2. By serial transfers of several strains of staphylococci and of a strain of enterococcus in broth and/or on the surface of blood agar containing increasing concentrations of erythromycin or streptomycin, high degrees of resistance developed against the homologous antibiotic, but cross-resistance to the other agent did not develop. Cross-resistance to neomycin was demonstrated in the strains of staphylococci which had increased in resistance to streptomycin following subcultures with the latter agent used either alone or in combination with erythromycin; such cross-resistance to neomycin did not occur in the strains which had become resistant to erythromycin following exposures to that antibiotic alone. The biological characteristics of the staphylococci, including coagulase production, remained unaltered following the development of resistance to either streptomycin or erythromycin, except for decreased production of pigment and less active growth of the resistant strains. The development of resistance to both erythromycin and streptomycin was delayed and depressed when the organisms were repeatedly subcultured in the combination of the 2 antibiotics. 3. It is concluded that the development of resistance to penicillin, streptomycin or erythromycin *in vitro* may be delayed and depressed by the use of a combination of erythromycin with either penicillin or streptomycin.

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Chloride Exchange between Human Erythrocytes and Plasma Studied with Cl^{36}.* (20043)

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It has been demonstrated by the use of isotope technics that in human blood sodium and potassium constantly exchange between erythrocytes and plasma under simulated physiologic conditions *in vitro*(1-6). This exchange is partially controlled by chemical processes which are very susceptible to temperature change and to some metabolic poisons. With this in mind, the exchange of chloride between plasma and erythrocytes has been investigated to determine whether the presence of similar metabolic control of the exchange of this anion could be demonstrated. In these experiments, Cl^{36} was added to whole blood, and the influence of changes in temperature and of several enzyme inhibitors on the speed of chloride uptake by the red cells measured. The inhibitors tested were NaCN, $NaNO_2$, Na-mono-iodoacetate, NaF, $HgCl_2$, Na-pyrophosphate, and sulfanilamide.

Methods. Heparinized venous blood was obtained from healthy donors, and 10 ml placed in paraffined flasks exposed to air at room temperature. After 30 minutes or longer, Cl^{36}, in 0.04 ml of saline solution, was

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TABLE I. Results of All Measurements of Chloride Exchange between Human Erythrocytes and Plasma.

Exp. No.		Temp., °C	Conc. of added compounds	Duration of exp.		Specific activity (Cl^{36}) of plasma as % of whole blood specific activity
				Min.	Sec.	
I		23	0	5		96.2
				12		99.9
				20		94.2
				40		99.2
II		21	0	2	20	92.8
III	a	38	0	2	35	102.2
	b	"	0	3	7	101.9
	c	"	NaCN 10^{-3}M	2	17	94.7
	d	"	$NaNO_2$ 1.5×10^{-2}M	2	27	95.2
	e	"	Na-mono-iodoacetate 10^{-3}M	2	25	95.4
	f	"	NaF 10^{-2}M	2	20	94.8
	g	"	$HgCl_2$ 10^{-3}M	2	30	100.3
	h	"	Na-pyrophosphate 10^{-3}M	2	38	98.7
	i	"	Sulfanilamide 2×10^{-2}M	2	35	96.1
IV		2–6	0	2	38	98.2
					Mean	97.3

added to the blood and mixing achieved manually by vigorous rotation of the flask for 30 seconds. Enzyme inhibitors were added 5-10 minutes before the isotope. After mixing, 5 ml of blood was removed and centrifuged briefly so that plasma could be separated approximately 2.5 minutes after adding Cl^{36} to the blood, and its radioactivity measured by methods previously described(7). After 3-4 minutes of additional centrifugation, plasma for chloride determination by the Schales technic(8) was obtained. The remaining whole blood in the flask was hemolyzed by freezing and similarly analyzed for radioactivity and chloride content. When the specific activity of the plasma had fallen to that of the whole blood, sufficient chloride must have moved in and out of the red cells to raise their specific activity to that of the plasma. Using formulations discussed in the literature(1), it was possible to estimate the lower limit of chloride transfer necessary to achieve this state. Since in all experiments there was no detectable difference in whole blood and plasma specific activity by the time they were first measured, at least 4 (probably more) T½ periods(1) were considered to have elapsed from the time of adding Cl^{36} until separation of the plasma.

Results. The results of all experiments and the conditions under which chloride exchange was measured are summarized in Table I. In Exp. III-c, plasma was collected most rapidly of all experiments and showed essentially the same specific activity as the whole blood. Calculation of the minimal chloride exchange rate in this instance showed that at least 95 g of chloride entered per liter of red cells per hour, or an amount equal to 50 times the red cell chloride content. In the several experiments, the exchange was not detectably different at 38°C, 21°C, or 2° to 6°C. Also, the addition of enzyme inhibitors and the conversion of hemoglobin to methemoglobin with sodium nitrite did not detectably alter chloride exchange.

Discussion. These data are in agreement with the qualitative results of others(9,10) and indicate that the rate of exchange of chloride between red cells and plasma is at least 600 and 1300 times more rapid than that of sodium and potassium respectively. The true difference may be very much greater, but the conditions of these experiments offered technical difficulties which made it impossible to measure the true speed of chloride exchange. In view of the ability of the red cells to affect the "chloride shift," the access of chloride to the cytoplasm of these cells may be controlled by conditions different from those in other body cells. An illustration of such a difference between cells is given by the unusual ability of those of the ileum and kidney to absorb chloride against concentration gradients. While this process may not involve control of chloride transfer directly, it is associated with metabolic processes that can be inhibited by added chemical compounds (11,12).

The experiments reported here do not suggest the presence of any metabolic processes controlling the exchange of chloride between erythrocytes and plasma, but they do not eliminate this possibility. They are consistent with the hypothesis that chloride enters red cells by passive diffusion. Approximate calculations show that the thickness of the plasma layer surrounding each erythrocyte in whole blood is about one micron, and that the red cell surface area in the 10 ml samples of blood used totals approximately 6 m^2, so that ample opportunity for rapid diffusion exists. However, because the actual rate of chloride transfer could not be measured, and because all possible metabolic systems were not inhibited, the results are not conclusive.

Summary. The rate of transfer of chloride into and out of human erythrocytes was estimated *in vitro* with Cl^{36} and found to be at least 600 to 1300 times greater than that of sodium and potassium respectively. Incubation at low temperature, the conversion of hemoglobin to methemoglobin, and the addition of metabolic poisons, were all without detectable effect on the rate of transfer. These data are compatible with the hypothesis that chloride enters erythrocytes by passive diffusion, but they do not eliminate the possibility that metabolic processes responsible for Cl transfer may exist which are capable of transporting chloride into and out of the red cell at a very rapid rate.

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A Pneumotropic Virus Isolated from C_3H Mice Carrying the Bittner Milk Agent. (20044)

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In a preliminary report(1), account was given of a virus isolated from C_3H mice known to carry the Bittner Milk Agent. This virus kills young suckling mice although inoculation of older suckling and of adult mice results in no obvious disease. Pathologic findings, as fully described elsewhere(2) are limited largely to the lungs regardless how the virus is inoculated and are different from those encountered in other types of mouse pneumonitis known to us. They consist primarily of swelling and proliferation of endothelial cells lining small blood vessels. The nuclei are swollen and contain inclusion bodies staining

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positively by the nuclear Feulgen procedure.

As this pneumotropic virus has thus far shown no relationship to other viruses, it is regarded as a hitherto undescribed agent, provisionally referred to as K-virus. Whether it is the Bittner Milk Agent or associated with it in any way has not been determined. Experiments have been set up, however, in conjunction with Dr. Howard B. Andervont, in an effort to answer this problem. Regardless of what relationships K-virus may prove to have on further study, its unique properties make it a subject of interest. The present communication describes further isolations of K-virus, production of specific immune serum, differentiation of K-virus from other viruses by neutralization test, titrations of virus in ill mice, and other properties not previously described.

Isolations of virus. Five isolations of K-virus in a total of 22 attempts made from female C_3H mice of a strain known to carry the Bittner Milk Agent,† have all caused illness on first passage in Swiss mice inoculated at one to 3 days of age. No blind passages have been resorted to. The first isolation, referred to as Lot 1, was from pooled liver, spleen, and tumors of 2 non-pregnant, non-lactating C_3H mice(1). Four other attempts to isolate virus from C_3H mice bearing tumors were wholly unsuccessful. The remaining 4 isolations (Lots 43, 47, 61, and 63) were from lactating mice, with litters of from 14 to 18 days of age which were removed the night before the mothers were sacrificed. Liver, spleen, and kidneys were pooled in each case and a separate pool made of mammary gland tissue. Such pools were emulsified in 10% meat infusion broth and inoculated intracerebrally and intraperitoneally in doses of 0.03 cc and 0.05 cc respectively. Illness first appeared within 9 to 12 days. In 2 of the mother mice, K-virus was isolated from mammary gland tissue as well as from liver, spleen and kidneys. All of the mice from which K-virus was originally isolated appeared well and had healthy young. K-virus, however, was present in the liver, spleen, and kidney pool of one mother in a titer of 10^{-4} and in the mammary tissue of the same mouse in a titer of 10^{-1} when these pools were assayed by intracerebral inoculation of 3-day-old mice. Of 5 C_3H mice 6 months of age nursing their first litters, K-virus was isolated from 3. Lot 47 virus was isolated from a mouse having her second litter. Twelve attempts to isolate K-virus from mothers with their third or fourth litters were unsuccessful. In the course of 15 passages with Lot 1 virus and 8 passages of Lot 43 virus no change in the pathogenicity of K-virus has been noted. The 5 viruses isolated appear to be similar in pathogenic effects and, as described below, were neutralized by the same antisera.

The disease picture in mice infected with K-virus remained the same in original as in later passages, regardless of route of inoculation. Inoculated mice grew normally and remained well-nourished until they became ill more or less abruptly. Even when moribund, they had milk in their stomachs. The principal manifestation of illness was labored "chugging" respiration, afflicted mice usually dying within 5 hours of its appearance. About 50% of young suckling mice had gross pulmonary consolidation, accompanied in some with an increase of pleural fluid. On histologic section, however, lungs of ill mice all presented a striking pathology(2). Altogether, 60 unsuccessful attempts to isolate virus have been made. A wide variety of mice have been tried including male and female C_3H, C-strain, and hybrid mice; all known to carry the *Bittner* Milk Agent; C_3H mice known to be free of the agent, and in addition Swiss mice, using pools of liver and spleen in most instances and tumor tissue or blood alone in others.

Susceptibility of mice and routes of inoculation. K-virus is infectious for suckling mice by a variety of routes including intranasal, subcutaneous, intraperitoneal, intracranial, and by stomach tube. Of these, only the last 2 have been employed at all routinely. The stomach tube was a 22-gauge needle with an extra blunt, slightly curved tip(3). In one experiment titrations of a pool of liver, spleen, and kidney by 3 different routes of inoculation were compared. Approximate titers were 10^{-4} by stomach tube, 10^{-7} by intraperitoneal, and 10^{-8} by intracerebral inoculation. The latter route was the most sensitive in spite of the fact that the inoculum used was .03 ml, as compared with .05 ml for intraperitoneal inoculation and 0.1 ml for the stomach tube; these amounts being those routinely employed. In another experiment Lot 43 virus was given to successively older mice both intracerebrally and intraperitoneally in an attempt to determine at what age susceptibility to apparent infection was lost. Resistance first definitely appeared in mice 12 days of age, but it was only in mice 18 days of age that resistance was complete, as no illness or deaths occurred in this group. Two mice survived in the 8-day group, but this was somewhat unusual as survivors have not been encountered in other experiments in which mice of this age were used. Although they never appeared ill, K-virus was readily recovered from a pool of liver, spleen, and kidney made from this latter group of mice 24 days after they had been inoculated.

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† Mice used for isolation of K-virus were kindly supplied by Dr. Howard B. Andervont, National Cancer Institute, National Institutes of Health, Bethesda, Md.

Distribution of virus in infected mice. To determine the distribution of K-virus in diseased mice, a pool of liver and spleen from first passage of Lot 43 virus was passed to 3-day mice intraperitoneally. Five pools of infective material were harvested from these second passage mice when they were moribund 8 days later. These pools were of blood, lungs, brains, intestinal contents, and finally of liver, spleen, and kidneys combined. Intestinal contents were collected by excising and chilling lower intestinal tract at 4°C, then pressing out the contents. Each pool was frozen in dry ice, thawed, then centrifuged at 4000 rpm for ½ hour prior to making serial dilutions for titration in brains of 3-day-old mice. Deaths occurring within 5 days were considered non-specific. All titrations were discontinued in 18 days although an occasional mouse inoculated with higher dilutions of virus died after this time. Continued spot checks indicated that mice became moribund while well-nourished, that they had a "chugging" respiration, and that at least 50% had gross pulmonary consolidation, these features being characteristic of infection with K-virus. Highest concentrations of virus (10^{-8}) occurred in lungs and in the pool of liver, spleen, and kidneys. Brains titered 10^{-5} and intestinal contents 10^{-6}. Blood from the original pool titered 10^{-4}, but due to loss of material, the titration had to be continued with blood from other mice similarly inoculated. This blood titered 10^{-7}. Bladder urine from a sick mouse produced typical illness in 3 of 6 mice inoculated intracerebrally with 0.02 ml. Subsequent experiments have shown that lungs as well as pools of liver, spleen, and kidneys from infected mice will not infrequently have an intracerebral titer of 10^{-9}.

To determine the rate of growth of K-virus nearly a million LD_{50} doses of virus were given to 3-day-old mice by intracerebral inoculation. Lungs and pools of liver, spleen, and kidneys of small groups of these mice were harvested one, 4, and 6 days later, as well as on the 7th day when they were ill and dying. Within 24 hours of inoculation, lungs had an infectivity titer of 10^{-2} and the liver, spleen, and kidney pool of 10^{-3} on titration in brains of suckling mice. Virus titers rose steadily and were maximal from tissues of those animals moribund on the 7th day, at which time tissues from both pools titrated 10^{-9}. Results on the 4th day indicated that growth of virus took place somewhat more rapidly in the lungs.

Neutralization tests. Good antisera to Lot 1 and Lot 43 viruses were produced in rabbits when pooled liver, spleen, kidney, lungs, and brains of infected mice, treated by differential centrifugation, were used as antigen. Ten per cent tissue suspensions were centrifuged at 4,500 rpm for ½ hour and the supernate recentrifuged at 15,000 rpm for one hour. The final sediment, suspended 1:10 in saline, was injected intravenously into 1800 g rabbits, 3 doses of 1-2 cc each being given at weekly intervals. Rabbits were bled 7 to 14 days after the last injection. In neutralization tests, sera were inactivated at 56°C for ½ hour and final mixtures contained equal amounts of serum and virus dilutions which were incubated not longer than ½ hour. Neutralization tests were carried out by intracerebral inoculation of 3-day-old Swiss mice. Rabbit serum prepared against Lot 1 virus neutralized 100 LD_{50} doses of K-virus in a

TABLE I. Results of Neutralization Tests in Which Different Lots of K-Virus Were Run against A) Specific K-Virus Immune Rabbit Sera and B) Antisera Prepared against Other Viral Pathogens of Mice.

	Sera	Time in days when all mice dead	K-virus lot used	Virus dilutions		
				1:20	10^{-2}	10^{-3}
A	Normal rabbit	8	Lot 47	ND	ND	7/7
	Lot 1 immune		"	0/9	0/8	0/6
	Normal rabbit	10	43	ND	ND	9/9
	Lot 1 immune		"	0/9	0/8	0/9
	Normal rabbit	10	I	ND	ND	9/9
	Lot 1 immune		"	ND	0/8	0/8
	Normal rabbit		43	ND	ND	8/8
	Lot 43 immune		"	0/8	ND	ND
B	NDV	14	43	ND	ND	8/8
	LCM	9	"			8/8
	PVM	12	"			8/8
	Coxsackie A*	10	"			9/9
	Coxsackie B†	11	"			9/9

* Pool of mouse antisera representing 10 types in Group A.
† " " " " " 4 " " " B.
ND = Not done.

dilution of 1:20. Serum prepared against the more virulent Lot 43 virus, however, fully neutralized an equal amount of virus in a dilution of 1:320. All viruses isolated in dilutions of 1:20 were neutralized by the same K-virus hyperimmune rabbit sera (Table I). Normal pre-inoculation rabbit serum failed to protect against the same virus preparations diluted 10^{-3}. A variety of other sera, which served as controls and failed to neutralize K-virus are divisible into 3 groups. The first group consisted of specific immune sera[‡] prepared against other viral agents of mice which might in any way be confused with K-virus (Table I). These sera include pneumonia virus of mice (PVM),[§] lymphocytic choriomeningitis (LCM), Newcastle disease (NDV), a pool of mouse antisera representing 10 types of Group A Coxsackie viruses and finally a pool representing antisera to 4 types of Group B Coxsackie viruses.[‖] A second group of sera were from normal adult mice of C_3H strain known to carry and of others known to be free of the Bittner Milk Agent, and from regular Swiss and C57 (black) mice. Human sera made a final group which included gamma globulin and sera from 2 of us (L.K. and H.W.M.) who have had laboratory exposure to K-virus. None of the sera in any of these 3 groups neutralized K-virus in final dilution of 10^{-3}, which represented 100 to 1000 LD_{50} doses of virus in these tests. Although naturally occurring antibodies have not been encountered in adult mice, continuing studies indicate that they may develop naturally in older suckling C_3H mice which never show signs of illness.

Physical properties. As discussed in the preliminary report(1), K-virus is resistant to freezing and thawing, to exposure to room temperature for 16 days and to penicillin and streptomycin and it passes readily through a Selas 03 bacteria tight filter. K-virus also resists 30 minutes exposure to 20% ether.

Discussion. Evidence presented above suggests that K-virus is an undescribed pathogen of mice. Its behavior in killing suckling but failing to cause illness or lesions in adult mice is not, to our knowledge, characteristic of other known mouse viruses. Coxsackie viruses behave in somewhat similar fashion but are not regarded as of murine origin. So far there is no evidence that K-virus belongs to this group, as it does not produce any sign of neuro-muscular changes nor do Coxsackie viruses lead to pulmonary consolidation. Furthermore, Coxsackie antisera from 14 different types in Group A and Group B have failed to neutralize K-virus. A special effort has been made to determine whether K-virus might be related to the Psittacosis-Lymphogranuloma group. There is no relationship on pathologic grounds, as LCL bodies do not appear in K-virus infected tissue. Centrifugation experiments indicate that K-virus is probably smaller than any member of the psittacosis group. Furthermore, complement fixing antibodies for the psittacosis-LGV group have not been found in K-virus immune serum. Antisera to other viruses infecting mice such as PVM, LCM, and NDV fail to neutralize K-virus, which resembles none of them in the type of disease produced. On results of serologic tests, disease pattern, and on the type of endothelial proliferation seen on histologic section of infected lungs(2), it would appear that K-virus is a previously undescribed agent. Our interest has centered largely in its ability to produce acute illness. A question of importance, however, is whether latent infection with K-virus can, in later life of its murine host, lead to mammary-adenocarcinoma.

[‡] As neutralizing sera against the Psittacosis-Lymphogranuloma group are not readily available, a complement-fixation test with K-virus immune rabbit serum was kindly run by Dr. M. R. Hilleman of the Army Medical Service Graduate School. Control and diagnostic antigens both gave a titer of 1:20, indicating that relationship of K-virus to the Psittacosis group is unlikely.

[§] Kindly supplied by Dr. Frank L. Horsfall, Hospital of the Rockefeller Institute for Medical Research, New York City.

[‖] Kindly supplied by Dr. R. J. Huebner from the Laboratory of Infectious Diseases, National Microbiological Institute, Bethesda, Md.

Longer term experiments essential to answering this question have been kindly set up by Dr. Howard K. Andervont. At the present time, in absence of proof from definitive experiments, the authors do not claim that K-virus is identical with the Bittner Milk Factor. It is hoped that further investigations will throw more light on the pathogenesis, mode of spread, immunology and relationships of K-virus infections in mice.

Summary. 1. Five isolations of a virus which causes a fatal pneumonitis of suckling mice by various routes of inoculation have been made from C_3H mice known to carry the Bittner Milk Agent. 2. Older suckling mice are increasingly resistant to this virus and no apparent illness has been produced by inoculation of adult mice. 3. Specific neutralizing antisera have been produced in rabbits. 4. Due to its unique behavior and failure to show serologic or other relationships to known mouse viruses, K-virus has been regarded as an hitherto undescribed agent. 5. Long term experiments have been set up to determine, if possible, whether K-virus is identical with or related to the Bittner Milk Agent.

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Occurrence of Generalized Infection of Enteric Origin in Mice Poisoned with Nitrogen Mustard.* (20045)

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The toxic effects of the nitrogen mustards on experimental animals include a number which resemble those produced by ionizing radiation(1-8). No observations seem to have been made, however, on the occurrence in animals poisoned with nitrogen mustard of spontaneous systemic infections such as have been found after exposure to moderate doses of total body x-radiation. For example: Miller, Hammond and Tompkins(9), described a high incidence of bacteremia in mice irradiated with 450 or 600 r during the second week post-irradiation, the period of greatest mortality.

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* This investigation was carried out under a contract between the U. S. Atomic Energy Commission and the University of Chicago.

Materials and methods. Methyl bis β Chloroethyl) amine hydrochloride† (HN-2) was dissolved in sterile distilled water and immediately injected intraperitoneally into Rockland Farms female RAP mice, 9 to 10 weeks old. Twenty minutes after the solution was made, any which remained was discarded and fresh solution was prepared. Preliminary experiments with doses ranging from .06 to .25 mg showed the LD_{90} (20 days) to be 0.2 mg. This dose, (0.2 mg) in 0.5 ml was used throughout the following experiments which included 610 mice, 258 of which were killed for culture. The mice had been kept under observation for one week after their arrival in the laboratory. Following injection they were housed either in groups of 20 in cages measuring 8 x 12 x 9 inches, or in groups of 30 in cages measuring 18 x 12 x 7 inches. The temperature of the mouse room was maintained at 73° to 78°F. The humidity was not controlled. They were fed a diet of mouse pellets made by Derwood Mills, according to a formula prescribed by the Argonne National Laboratory. Their water bottles were changed daily. After treatment with HN-2, 5 to 10 mice were sacrificed each day for culture of heart's blood and spleen. The mice were etherized and autopsied under aseptic conditions. As much blood as possible (0.5 to 1.0 ml) was drawn from the heart into a capillary pipette. One drop was cultured on the surfaces of a blood agar plate and an eosin-methylene blue plate, and the remainder in brain-heart infusion broth. No anaerobic cultures were made because experience had shown that mice of this strain carry very few anaerobes in their intestinal tracts, and because anaerobes were so rarely recovered from the heart's blood of mice subjected to total body irradiation(9). The spleen was removed, cut in several places and dropped into 5 ml of brain-heart infusion broth. The cultures were incubated at 37°C and examined each day. At the first appearance of bacterial growth, the cultures were streaked onto blood agar and EMB plates and the micro-organisms identified by methods previously described (9). All negative cultures were incubated for 72 hours, at the end of which time they were subcultured onto blood agar and EMB plates before they were discarded. *Mortality controls.* To obtain data on the death rate of mice receiving this dose of the drug, 2 groups of mice, totaling 144, were injected with 0.2 mg of HN-2 and observed for twenty days. The deaths occurring each day (plotted as per cent of the number of mice alive at the beginning of that day) are shown in Fig. 1.

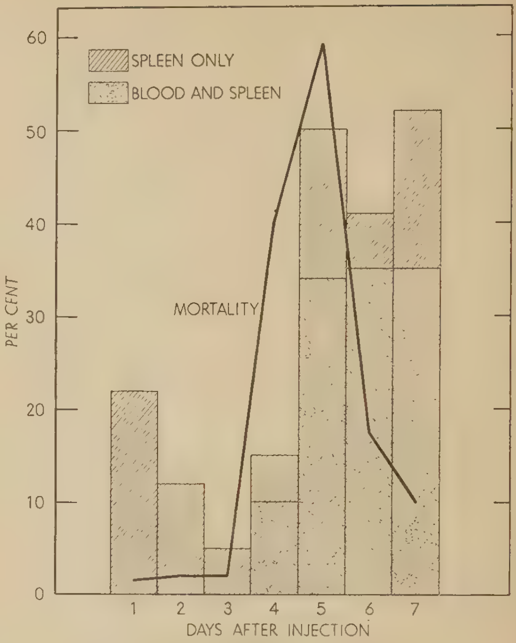

FIG. 1.
Incidence of positive cultures and mortality.

Results of blood and spleen cultures. Table I shows the number of mice from which positive cultures of blood and/or spleen were obtained each day in each series. The figures in parentheses indicate the number with positive spleen and negative blood cultures. Every mouse with a positive blood culture had a positive spleen culture as well. All but nine (13%) of the cultures were pure cultures. It will be noted that in the first three series, 18 of the 37 positive cultures contained *Salmonella enteriditis.* This disproportionately high incidence can be explained by the fact that the mice used at that time were found to contain an unusually large number of Salmonella carriers.

A diagrammatic summary of the results is

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† Merck & Co., Rahway, N. J.

TABLE I. Daily Record of Positive Cultures of Blood and/or Spleen.

Date of inj.	No. of mice	Days after IP injection of .2 mg HN-2 1	2	3	4	5	6	7	8
4–10–51	130	(2)†/10*	4(3)/10	0/10	0/5		3(1)/10	6(3)/10	1/5
20	100	(2)†/5	0/5	0/10	(1)/10	7(4)/11			
26	50	(1)†/5			0/5	2(1)/8	5/8		
5–29	15					3/6			
1– 9–52	10		0/5						
16	50	0/5	0/5	(1)/5		4(1)/8			
29	70	(2)/5	(1)/5	0/5	0/5		2(1)/5		
31	50				2/5	3/5	3/5		
3– 4	80	(1)/5	0/5	0/5	(1)/5		1/6	3/7	
24	55	(1)/5	0/5	(1)/5	2/5				2(1)/4
Total mice	610	40	40	40	40	38	34	17	9
% positive		22	12	5	15	50	41	52	33

* Denominator = No. of mice killed and cultured. Numerator = No. of mice yielding positive blood and/or spleen cultures. Parentheses = No. of these mice yielding positive spleen cultures only.

† The positive cultures in the first 3 series included 18 containing *Salmonella enteriditis*.

TABLE II. Micro-Organisms Recovered from Heart's Blood and/or Spleen.

Salmonella enteriditis	39%*
E. coli	25
Proteus	20
Paracolobactrum	16
Pseudomonas aeruginosa	4
C. pseudotuberculosis murium	4
α Streptococci	1

* % of all strains recovered. Positive cultures of both heart's blood and spleen from a single mouse were counted as one strain.

presented in Fig. 1 which shows that a high incidence of bacteremia (33-35%) occurred during the 5th, 6th and 7th days. During this period, a number of additional mice (6-17%) were found to have bacteria in their spleens. A fairly high percentage of the mice killed in the first 2 days were also found to have infected spleens.

Bacteria obtained from blood and spleen cultures. A classification of the bacteria recovered from cultures of heart's blood and spleen is presented in Table II. All except *Corynebacterium pseudotuberculosis murium* have been found to be normal inhabitants of the intestinal tract of this strain of mice. As already mentioned, most of the cultures of Salmonella were obtained from the mice in the first 3 series which contained a high incidence of carriers of this micro-organism.

Discussion. These experiments demonstrate the development of generalized systemic infection in a considerable number of mice which had been poisoned with nitrogen mustard and killed for culture of heart's blood and spleen. It should be pointed out that mice which died spontaneously were discarded without culture. The maximum incidence of bacteremia occurred on the 5th, 6th and 7th days, much earlier than in mice subjected to total body irradiation(9). This period did not coincide exactly with the period of greatest mortality as it did in the irradiated mice. However, the data for the nitrogen mustard mortality curves were not obtained from mice which were cultured, as in the case of irradiated mice, but were obtained from two groups which were given nitrogen mustard solely for the purpose of determining the daily death rate. A much larger proportion of mice poisoned with nitrogen mustard was found to have positive spleen (and negative blood) cultures than was the case in the irradiated mice, particularly during the first two days.

As in the case of the irradiated mice, the bacteria recovered from heart's blood and spleen were all members of the normal intestinal flora with the possible exception of *Corynebacterium pseudotuberculosis murium* which was not encountered in any cultures of the intestinal content of these mice.

It must be emphasized that the data herein reported are based on mice poisoned with an LD_{90} of nitrogen mustard. No therapeutic experiments were made to determine whether it would be possible to control the development of bacteremia by administering suitable antibiotics, and if so, whether such treatment would significantly reduce the mortality.

Korol(10) obtained equivocal results in one experiment in which mice poisoned with nitrogen mustard were treated with streptomycin, but the treatment was not continued beyond the 4th day. In one unpublished experiment Korol(11) found a slight reduction in mortality in mice treated for 12 days with 7000 μg of streptomycin a day.

While the development of bacteremia may hasten the death of animals poisoned with nitrogen mustard, it seems doubtful that its importance as the cause of death is as great as it appears to be after moderate doses of total body x-radiation.

Summary. 1. Nine to 10 weeks old female mice, averaging 27 g in weight were injected intraperitoneally with 0.2 mg (approximately 7 mg per kilo = LD_{90}) of nitrogen mustard (Methyl bis (β-chloroethyl) hydrochloride). Cultures of heart's blood and spleen of mice sacrificed for that purpose were made at daily intervals. 2. Bacteremia was found to be present in over $\frac{1}{3}$ of the mice on the 5th, 6th and 7th days. A number of additional mice on these days (6-17%) showed positive cultures of spleen only. The species of bacteria recovered from blood and/or spleen were all members of the normal intestinal flora, with the exception of *Corynebacterium pseudotuberculosis murium*. 3. Generalized infection of enteric origin seems to play a less important role as a cause of death in mice poisoned with nitrogen mustard than in mice subjected to moderate doses of total body x-radiation.

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Growth Stimulation of *Lactobacillus gayoni* by N-D-Glucosylglycine.*

(20046)

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(Introduced by R. Wulzen.)
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Early studies on the nutrition of *Lactobacillus gayoni*(1) provided evidence for a "gayoni factor", inasmuch as this organism could not be cultured on existing synthetic media unless supplemented by liver or yeast fractions. Subsequent experiments(2,3) showed that yeast nucleotides and unusually high levels of pteroylglutamic acid were required in addition to the active principle of liver or yeast. Recently, it has been found possible to replace in part the nutrients in liver or yeast by glutamine or asparagine, although maximum growth could not be obtained(4).

The growth of *L. gayoni* also has been found to be dependent upon heating the complete

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TABLE I. *L. gayoni* Response to Asparagine and Yeast Extract as Influenced by Heating the Culture Medium and by Supplements of Ascorbic Acid.

Test materials	Filter-sterilized medium*		Heat-sterilized medium*	
	Control	Ascorbic acid (4 mg/10 ml)	Control	Ascorbic acid (4 mg/10 ml)
	Growth response (O.D.)†			
None	.08	.10	.12	.16
Asparagine (10 mg/10 ml)	.19	.23	.73	.89
Yeast extr. (50 mg/10 ml)	.79	1.02	1.17	1.24

* Culture medium minus asparagine and ascorbic acid. Heat sterilization—10 min. at 120°C 50 mg of yeast extr. = ca. .5 mg of asparagine. *L. gayoni* asparagine requirement = .1–.3 mg per 10 ml.

† O.D. (optical density) = 2-log % transmission. Each value is average of 2 determinations. Time—12 hr at 37°C.

culture medium. Heating the components of the medium separately and mixing after cooling or filter sterilizing the medium did not provide for any stimulatory factor. This factor could also be supplied by high levels of a yeast extract.

Procedure. The test organism was strain 45 of *L. gayoni* 8289 which was maintained according to previously described procedures (2,3). The culture medium of Cheldelin and Nygaard(2) was employed with supplements of ammonia-hydrolyzed yeast nucleic acid[†] (Schwarz) (1 mg/10 ml), L-asparagine • H_2O (1 mg/10 ml), ascorbic acid (4 mg/10 ml), xanthine (0.1 mg/10 ml) and pyridoxal • HCl (10 γ/10 ml). The previously listed concentrations of salts A and Tween 80 were increased 3-fold. Such a medium was complete enough to allow for maximum growth within 20 hours of incubation, even when unheated. However, for a maximal growth rate during 10 to 14 hours of incubation, a large supplement of yeast extract (Difco) (ca. 50 mg/10 ml) was required. When the components of the culture medium were heated separately or in various combinations, each component was adjusted individually to pH 6.8 before heating. When heat sterilization was not applied, the medium and supplements were filter sterilized using either sintered glass or Seitz filters. N-D-*glucosylglycine* ethyl ester was prepared by condensing glucose with glycine ethyl ester according to the procedure of Wolfrom *et al.*(5) (m.p. 106.5-107.5°C;[‡] literature m.p. 108°C). Henceforth in this report, this ester will be referred to as GG. GG did not give a positive ninhydrin test under conditions where a positive test was obtained from free glycine. Neither was a positive reaction obtained by the aniline acid phthalate test for reducing sugars(6). Upon heating an aqueous solution of GG, positive tests were obtained for the free amino acid and reducing sugar. By *saponification* of GG with alcoholic KOH, the potassium salt was obtained in quantitative yield. This salt was isolated as a hygroscopic white powder which contained bound amino and reducing groups. When warmed with ninhydrin, the solution "browned" before giving a positive test for the free amino acid. The *alkaline treatment* of GG or glucose consisted of autoclaving 120 mg of material in 50 ml of one per cent potassium phosphate buffer (pH 9.5) for 10 minutes at 120°C. After cooling, the solution was adjusted to pH 6.8 and diluted to 60 ml. In certain experiments as noted, a few of the supplements were omitted from the basal medium and added as test materials.

Results. When the test organism was cultivated in an unheated medium, it did not grow as rapidly as it did when the medium had been heated (Table I). Although ascorbic acid provided some stimulation, especially in the more complete media, the effect of heating the medium was considerably greater.

In experiments which are not being reported here in detail, it was found that omitting the glucose or acid-hydrolyzed casein from the medium during heating reduced the heat activation effect. When only glucose and the

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[†] Employed as a source of the yeast nucleotides.

[‡] Uncorrected (Fisher-John block).

TABLE II. *L. gayoni* Response to the Substance Formed by Heating Together Various Components of the Culture Medium.

Components of medium heated together*	Growth response (O.D.)†
0 (all filter-sterilized)	.23
G + S	.27
G + S + casein hydrolysate	.51
glycine	.64
D,L-α-alanine	.12
β-alanine	.42
D,L-valine	.41
D,L-norvaline	.41
D,L-leucine	.34
D,L-isoleucine	.39
D,L-serine	.25
D,L-threonine	.36
L-cystine	.18
D,L-methionine	.22
L-lysine • HCl	.41
L-histidine • HCl	.39
L-arginine • HCl	.39
D,L-aspartic acid	.36
L-glutamic acid	.42
L-tyrosine	.35
D,L-phenylalanine	.30
D,L-tryptophane	.38
L-proline	.29
L-hydroxyproline	.25

* G = glucose (100 mg); S = salts A (.15 ml). All amino acids and casein hydrolysate were tested at 4 mg (except cystine which was tested at 2 mg). Total volume = .4 ml. Heating time—10 min. at 120°C.

† O.D. (optical density) = 2-log % transmission. Time—12 hr at 37°C.

TABLE III. *L. gayoni* Response to Graded Levels of N-D-Glucosylglycine, Ethyl Ester and Potassium Salt.

Test materials (mg/10 ml)		Growth response (O.D.)*
0		.32
Ethyl ester	.01	.35
	.1	.42
	1	.67
	10	.62
	100	.12
K salt	1	.37
	10	.50
	100	.05

* O.D. (optical density) = 2-log % transmission. Each value is average of 3 determinations. Time—13 hr at 37°C.

acid-hydrolyzed casein (previously adjusted to pH 6.8) were heated together, only slight amounts of activity were formed; the presence of salts A (potassium phosphates, pH 6.8) was required in order to obtain the full effect of heat activation. Heating glucose with salts A also resulted in only incomplete activation.

When the amino acids of the acid-hydrolyzed casein were treated individually with glucose and salts A, glycine was found to be the amino acid most effective for activation (Table II). Many of the other amino acids were slightly effective and some were inhibitory. In a subsequent experiment, only α-alanine was found to be inhibitory when heated with glucose, salts A and glycine.

Solutions of glucose, salts A and amino acids were observed to "brown" on heating and the possibility that activation was related to the Maillard reaction(7) was explored. Several intermediates of the "browning" reaction involving glucose and glycine were tested, among them N-D-glycosylglycine, as the ethyl ester and the potassium salt. These materials were found to be active when tested in the unheated medium (Table III), but the potassium salt was only about one per cent as active as the ester. When these materials were heated with the culture medium, the broth containing the potassium salt "browned" more than the broth containing the ester. Perhaps, a free carboxyl group favored destruction of activity.

The GG response curve deviated from that of yeast extract (Fig. 1). With increasing concentrations of GG, the response increased slowly at first, then progressively more rapidly than the yeast extract response. Ultimately, a maximum was reached and higher concentrations of GG inhibited growth. Neglecting these higher levels, where inhibition was evident, the GG curve was reminiscent of curves obtained with deficient media. Several limiting factors appeared to have been increasing simultaneously, or in other words the activity of GG may have represented multiple effects. When GG was autoclaved under alkaline conditions, an overall decrease in activity was observed. However, the response curve then paralleled the yeast extract curve and no inhibitions were observed at the high supplement levels. Subsequent tests have shown that although some activity was always destroyed by heating, prolonged heating (60 minutes instead of the 10 minutes employed here) did not reduce the activity further. These data were interpreted as indicating a

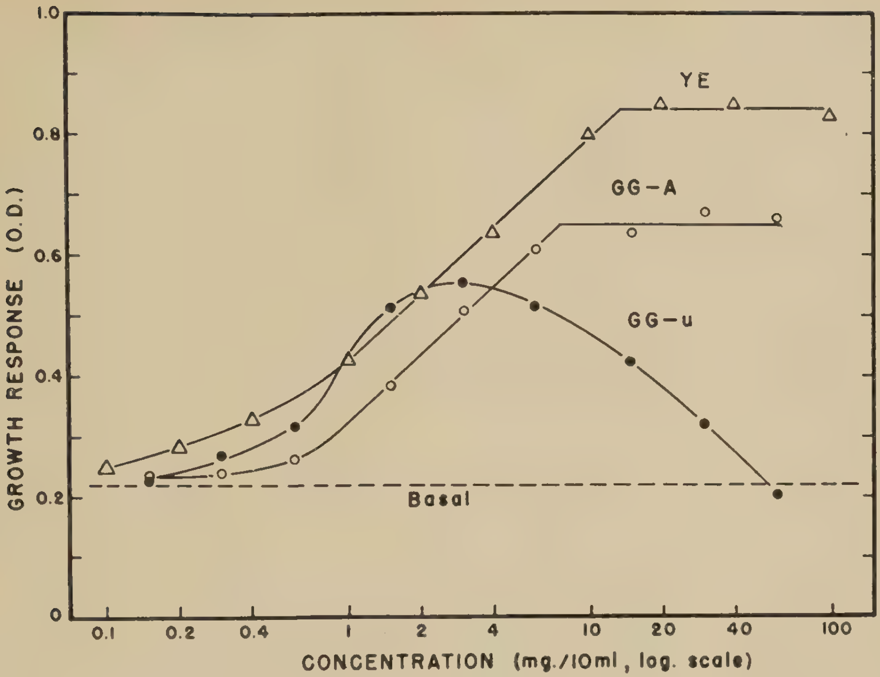

FIG. 1. *L. gayoni* response to graded levels of N-D-glucosylglycine ethyl ester, unheated (GG-U) and autoclaved at pH 9.5 (GG-A), and yeast extract (YE). Time—13.5 hr at 37°. Each point is average of 4 determinations.

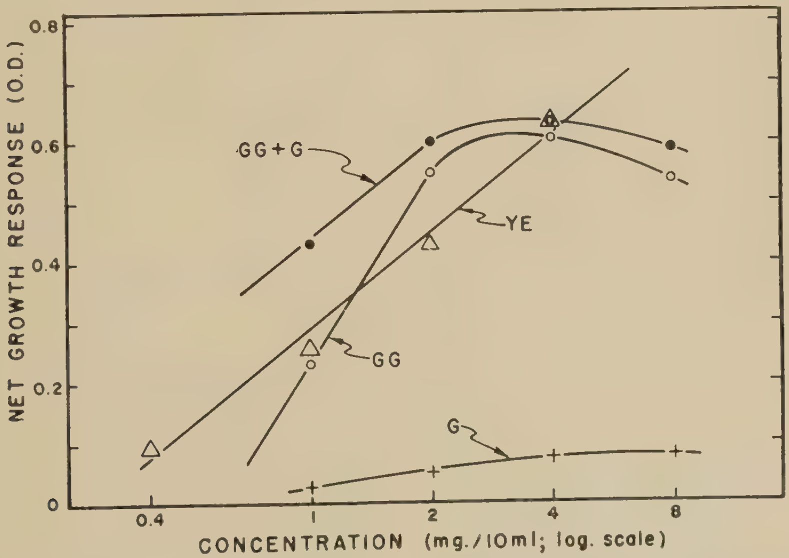

FIG. 2. *L. gayoni* response to graded levels of yeast extract (YE), N-D-glucosylglycine ethyl ester (GG) and glucose (G) autoclaved at pH 9.5. (GG + G) = graded levels of GG with constant amounts of G added to basal medium (4 mg/10 ml). Time—13 hr at 37°. Each point is average of two determinations.

conversion of GG into another, heat stable form, perhaps even more active. The initial loss of activity was apparently due to destruction of GG before it could be converted into the other form, and the total activity was regarded as a net effect of several competing reactions of destruction and synthesis.

Glucose was autoclaved under alkaline conditions to form a substance with very slight activity when tested alone on the unheated medium (Fig. 2). A 4 mg supplement/10 ml for instance, furnished a net culture turbidity of only 0.07 O.D. units. However, in the presence of this 4 mg supplement of autoclaved glucose, the growth response curve for GG, which without supplementation deviated from the yeast extract curve, became parallel to that of yeast extract. Evidence for synergisms between autoclaved glucose and low levels of GG (unheated) was striking. Toxicity at high levels of GG was again noted.

Discussion. The need for heat activation of the culture media employed for lactic acid bacteria was discovered by Orla-Jensen(8). Heat activation was found to involve substances like glucose, methylglyoxal, furfural, arabinose and xylose with the remainder of the culture medium, autolyzed yeast or even alkaline tap water. Snell *et al.*(9) have reported that pyruvate could replace the substance formed by heating the culture medium for *L. bulgaricus*. Under the present conditions however, pyruvate was totally without effect for *L. gayoni*. This suggests that the heat activation problem may be much more complex than is even indicated in these papers.

The possibility that these two postulated growth factors may be the ultimate solution to the "gayoni factor" is favored by the fact that the heated GG curve and the unheated GG plus heated glucose curve were parallel to the yeast extract curves (semi-log scale). However, definite assurance of the microbiological equivalence of these factors to yeast extract can not yet be given because of the superior total growth that is possible at high concentrations of yeast extract. The inadequacy of GG plus heated glucose may be due to toxic substances, to a marginal deficiency of some component of the culture medium, to other growth factors, or other more active forms of existing factors.

Summary. 1. For rapid initiation of growth of *L. gayoni*, the culture medium required heating. 2. This heat activation reaction, involving glucose, salts A and glycine, appeared to proceed through an intermediate, N-D-glucosylglycine, and then into at least two heat stable growth factors. 3. One of these factors could also be derived by heating glucose under alkaline conditions.

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Oxidative Dissimilation in Pantothenate-Deficient *Acetobacter suboxydans* Cells.* (20047)

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Work has been focused in this laboratory on the metabolism of *Acetobacter suboxydans*,

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because this organism preferentially uses conjugated forms of pantothenic acid [a pantothenic acid conjugate (PAC(1); coenzyme A (2,3)] over the free vitamin. Attempts to localize the action of pantothenic acid by checking reactions which are known to be coenzyme A dependent(4,5) were unsuccessful. Although hot water extracts of *A. suboxydans* cells contained coenzyme A, oxidation of acetate or any of the Krebs cycle intermediates failed. The organism was unable to form citrate or to acetylate sulfanilamide, even in the presence of external sources of coenzyme A and adenosine triphosphate.

However, preliminary experiments on the oxidation of glycerol revealed that oxygen consumption beyond one atom per molecule was occasionally accelerated by the addition of crude enzyme A to the well-washed resting cells. The results were not consistent, perhaps due to the difficulty of removing coenzyme A by the washing procedure, and the oxidation of various substrates was therefore studied in pantothenate-deficient cells. The present paper reports the retardation of some of these oxidations during pantothenate deficiency.

Experimental. A strain of *A. suboxydans,* ATCC No. 621 was used for the experiments. *Materials.* PAC(1), in a crude state containing some coenzyme A, was used as the pantothenic acid source for cell growth. This concentrate was used in preference to the free vitamin since the growth response was more nearly a linear function of the added nutrilite when these conjugates were used(1). A coenzyme A preparation of 75% purity, obtained from the Pabst Laboratories, was employed in the oxidative studies. *Pantothenic acid analysis.* This was carried out with *Lactobacillus arabinosus*(7) after enzymatic digestion(8). *Preparation of cells. A. suboxydans* cells were grown on 3 different levels of PAC in a synthetic medium described earlier(6). The inoculum was grown in 1500 ml of the medium with aeration for 43 hours. The cells were washed twice with sterile saline, and then transferred in equal amounts to three 5-gallon jars, each containing 8 liters of medium. To the mixtures (labeled A, B and C), the PAC-coenzyme A preparation was added at concentrations of 0.6, 5, and 50 γ pantothenic acid equivalent per liter of medium. These concentrations in a small scale preliminary experiment gave respectively 20, 67, and 100% of the maximum turbidity of culture. The jars were incubated for 48 hours with heavy aeration, at summer temperatures (25-30°C). After centrifugation and 3 washings (one in 0.067 M potassium phosphate buffer of pH 6.0, two in distilled water), the cells were dried *in vacuo* from the frozen state. They were then investigated for their dissimilation of various substrates by standard Warburg manometric technics.

Results and discussion. The yield of dry cells for samples A, B, and C were respectively 0.5, 1.1, and 0.9 g. Testing of the cells showed that cells C were not appreciably different from B in their oxidative capacity for either ethanol, glycerol or sorbitol. The experiments described below were therefore carried out only with the cells from the 2 lower levels of pantothenate, namely samples A and B.

As shown in Fig. 1, *alcohol was oxidized* rapidly by both cells A and B, with no significant difference in rate. The oxidation ceased after 80 minutes, at an oxygen consumption of 95% of the theoretical. The alcohol oxidation was thus independent of changes in the pantothenate content of the cells under the conditions used.

For *oxidation of glucose* the velocities also were the same for the two preparations, up to a consumption of one atom of oxygen per molecule substrate. At that point, however, the oxidation in the deficient cells leveled off sharply. With sorbitol, the initial oxidation rate was lower in the deficient cells, and the process virtually ceased when one atom of oxygen had been consumed. Sorbose was not oxidized when supplied as the exogenous substrate (data not shown in Fig. 1). These results indicated that coenzyme A was needed for the second step of glucose or sorbitol dissimilation, and possibly in the first step of sorbitol oxidation.

Similar behavior was exhibited toward *glycerol.* The reaction velocities were the same for cells A and B up to one atom of oxygen consumed per molecule of substrate.

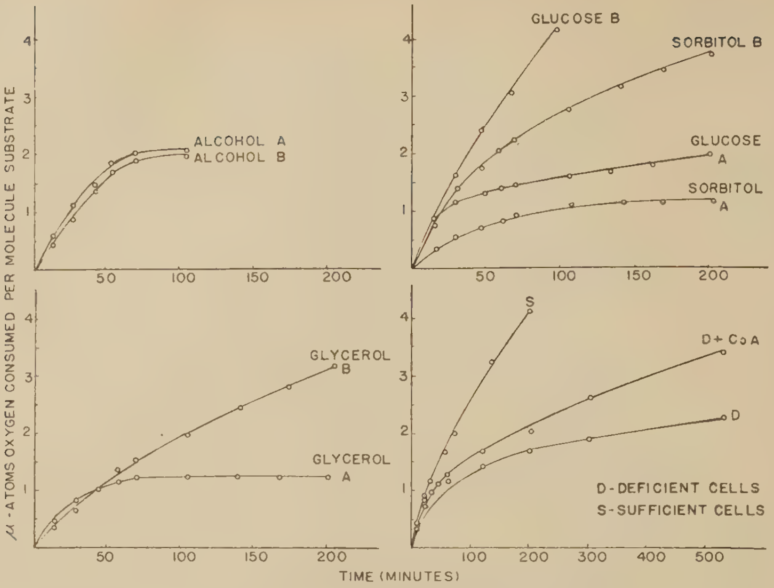

FIG. 1. Effect of pantothenate deficiency on oxidations in resting cells of *A. suboxydans*. Each vessel contained 20 μ moles sorbitol or glucose, or 40 micromoles of glycerol or alcohol; .05 M Sörensen's phosphate buffer at pH 6, .01 M $MgCl_2$, .3 ml of .5% diphosphopyridine nucleotide, and .2 ml of 20% KOH. 10 mg (dry wt) of *A. suboxydans* cells were used, and water added to 3 ml. The fourth group of curves (lower right) show the effect of coenzyme A on glycerol oxidation by resting *A. suboxydans* cells. Cells D (pantothenate deficient) contained 6.6 γ calcium pantothenate/g; cells S (pantothenate sufficient) contained 27 γ/g. Supplements were added as described in Fig. 1.

The rate of oxygen uptake by cells A then diminished sharply, whereas cells B proceeded to consume oxygen rapidly for several hours. This indicated an inability on the part of the pantothenate-deficient cells to oxidize dihydroxyacetone to any significant extent. A separate experiment with dihydroxyacetone as substrate was then conducted and confirmed this finding.

The fourth group of curves in Fig. 1 demonstrate the effect of exogenous coenzyme A upon glycerol oxidation, by a second sample of pantothenate-deficient cells. Although these cells possessed somewhat greater capacity for dihydroxyacetone oxidation than those described in the foregoing paragraphs, the addition of 500 γ coenzyme A per flask more than doubled the rate of oxygen consumption; for the first atom of oxygen consumed, the rate increase was 130%. It thus appears that the dissimilation of dihydroxyacetone in pantothenate-deficient cells is coenzyme A-linked, although it has not yet been possible to prepare a coenzyme A-dependent apoenzyme from the dihydroxyacetone oxidase. Further experiments are in progress to investigate these reactions in cell free systems.

Summary. 1. Resting cells of *A. suboxydans,* grown with deficient levels of pantothenic acid, were investigated for their ability to oxidize a series of substrates. These cells showed retarded ability to oxidize dihydroxyacetone, sorbose, and gluconic acid, and somewhat poorer ability to convert sorbitol to sorbose. 2. Addition of coenzyme A to the deficient cells significantly improved their ability to oxidize dihydroxyacetone, thus suggesting that the further dissimilation of this substrate is coenzyme A-linked. 3. The oxidation of alcohol, of glycerol to dihydroxyacetone, and of glucose to gluconic acid proceeded with equal velocity in deficient and normal cells, indicating non-involvement of coenzyme A in these reactions.

The authors are grateful to Mrs. Marion MacDonald for the large scale preparation of *A. suboxydans* cells.

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Factors Associated with the Incidence of Infantile Diarrhea in Mice.* (20048)

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Infantile diarrhea in man and other mammals is classified as an infectious disease but searches for common, specific agents have failed. Etiology of infantile diarrhea in mice has been attributed to a Salmonella microorganism in one instance(1) and a virus in another instance(2). Both reports further indicate that a variety of factors such as temperature, diet, season, infection, and physiological condition of parents and offspring may, singly or cumulatively, be influential in determining the severity of an epidemic. Infantile diarrhea is prevalent in many colonies of experimental mice and presents a serious handicap for standardizing experimental animals.

Methods and materials. A colony of C3H mice was observed for symptoms of diarrhea in suckling animals between September 14, 1949 and January 17, 1950. More than 900 animals were born during this period of which almost 30% displayed severe diarrhea before they were 18 days old. Symptoms of diarrhea varied from a mild condition recognizable only by the finding of fecal material adhering to the under surface of the tail, to the passage of large amounts of yellowish, watery, feces. The intensity of the disease varied within litters, but the onset of symptoms usually occurred between 8 and 14 days. The mice were kept under conditions typical in our laboratory, *i.e.,* 75°F, exhaust ventilation and water and Purina Fox Checkers *ad libitum.* Clean dry pens of wood were provided on Tuesday of each week. Individual pairs of breeders were kept together at all times. Notation concerning the presence of diarrhea was made on the parent's pedigree card. The subsequent analysis of data was made on the basis of litters as units. Correlations were made (a) between litters born just before and just after clean pens were supplied (b) between litters born to females of different ages (c) between litters of different parity (d) between litters of different size and (e) between litters born in 3 successive 5-week periods. Significance of differences in these correlations was tested by the χ^2 test for homogeneity.

Results. Data were obtained from 76 pairs of C3H mice and 900 young born to them during the period of 4 months (Table I). Thirty-seven of the pairs gave birth to 62 litters, including 350 young, none of which showed appreciable symptoms of diarrhea. The remaining 39 pairs had 92 litters, which included 550 young, of which 46 litters (50%) were discarded due to severe diarrhea. The overall incidence was about 30% of the young born. Although the incidence of diarrhea remains high the colony of animals still prospers. The following tabulations and cor-

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TABLE I. Summary of Observations Made on Litters Born between 9/14 and 1/7.

	No. of pairs of parents	No. of litters observed	No. of litters affected	%
Parents which had no diarrheal litters	37	62 (350 young)	0	—
Parents which had at least 1 diarrheal litter	39	92 (550 ")	46	50

relations are based on the 92 litters born to those 39 pairs of parents which had at least one litter with diarrhea during the observation period.

The first correlation was made by grouping the 92 litters according to the part of the week in which they were born, *i.e.*, before or after the weekly supply of clean boxes was put in the colony. This was done to see if the disease might be partially attributable to birth of young animals in established but dirty pens, or in pens recently disrupted but clean. The comparison showed that 56% and 50% of young contracted diarrhea in the 2 groups respectively, therefore no obvious influence of the relative condition of the nest was demonstrated.

Correlations made between incidence of diarrhea and the age of the parents (Table II) showed an inverse relationship between the two. Animals in litters of the younger parents (60 days to 120 days) had a greater tendency to contract the disease than did litters of parents over 120 days, 58% and 40% respectively. A second correlation was made between the incidence of diarrhea and the seriation of the litter (Table III). Here also an inverse relationship existed between the frequency of the disease and the factor being correlated; the young in litters of the early parities, 1 and 2, had a greater tendency to contract the disease than did those of later parities, 3 through 6. Thus it was demonstrated that a higher incidence of diarrhea occurred in litters born to young females and in early litters. As the seriation of the litter and the age of the parents are related, more data would be necessary to separate these two factors.

Correlations made between occurrence of diarrhea and size of litter (Table IV) indicated a direct relationship between the two; the percentage of the litters affected increased directly with their size. Half of the litters of 6 or more contracted diarrhea as compared to 28% of litters of less than 6 animals. The 3 foregoing observations indicate that the physiological condition of the parents, *viz.*, age, parity, and litter size, was implicated with the appearance of the disease.

TABLE II. Distribution of Diarrheal Litters in Relation to Age of Mother.

Age of parents (days)	No. of litters observed	No. of litters with diarrhea	% with diarrhea
60–90	31	18	58
90–120	19	11	
121–150	20	10	40
151–180	7	3	
181–200	3	2	
>200	12	2	

TABLE III. Distribution of Diarrheal Litters in Relation to Seriation of Litters.

Serial order of litters	No. of litters observed	No. of litters with diarrhea	% with diarrhea
1	27	15	58
2	25	16	
3	16	6	38
4	10	5	
5	9	3	
6	5	1	

TABLE IV. Distribution of Diarrheal Litters in Relation to Size of Litter.

Size of litter	No. of litters observed	No. of litters with diarrhea	%
3	8	3	28
4	7	0	
5	10	4	
6	17	7	50
7	11	5	
8	11	7	
9	5	3	
*	23	17	

* Not recorded at birth.

The 4-month period of observations was

TABLE V. No. of Diarrheal Litters during Each of Three Periods.

5-wk periods	No. of litters observed	No. of litters with diarrhea	% with diarrhea
1 (9/4 -10/22)	23	11	48
2 (10/23-11/29)	30	12	40
3 (11/30- 1/7)	39	23	59

TABLE VI. Frequency of Diarrhea in Litters during Periods 1 and 2 and 3; Comparisons with Size and Seriation of Litters and Age of Parents.

		Periods 1 and 2	Period 3
Size of litter	1–5 young	5/23†	2/7
	6–9 "	10/26	12/18
	*	8/9	9/14
Serial order of litters	1–2	15/31	16/21
	3–6	8/22	7/18
Age of parents (days)	60–120	13/30	10/23
	>120	16/20	7/19

* Not recorded at birth.

† Numerator = litters with diarrhea; denominator = litters observed.

divided into 3 portions of approximately 5 weeks each and the number of litters with and without diarrhea tabulated for each period. The results of the grouping are shown in Table V. There was an increase from 48 and 40% of the litters affected in each of the 2 early periods to 59% in the last period, *i.e.*, during December and the first week of January.

Discussion. The data collected for a 4-month period from a colony of C3H mice in which diarrhea was endemic indicated that the physiological condition of the parents, *viz.*, age of parents, seriation of litters, and size of litters, had influence upon the occurrence of diarrhea in their offspring. Increased incidence of diarrhea in December may have been due partially to birth of a larger proportion of a) early parities b) large litters and/or c) litters born to young females. Even when litters in early parities and from young parents were compared, the incidence of diarrhea was higher in December, period 3. Table VI shows that during this period there was also a higher incidence of the disease in litters born to older females and in litters of small size (3 young or less). A temporal factor evidently was present therefore, during the period observed, but this was superimposed upon other contributing factors which have been classified under physiological condition of parents. The higher incidence of the disease in December suggests that season might influence its expression. This may lead to reduction or elimination of the disease by more rigid control of the seasonal factors.

The condition of the pen in relation to cleanliness, as indicated by the part of the week during which the young were born, was found not to be associated with a difference in the incidence of diarrhea. The data show that neither the soiled condition of the pen nor the neglect of newborn at or near time of birth differentially affected the incidence of diarrhea.

Some factors possibly related to the disease which were not examined include change of food formula with new shipments, variations in the supply of bedding and introduction of infectious agents.

Summary. 1. Observations on 900 C3H mice born to 76 parents showed that 30% of the young contracted diarrhea severe enough to make it necessary that they be discarded. The 46 litters so affected comprised 50% of the offspring from 39 pairs of mice. 2. Observations on the 39 pairs of parents which had one or more litters afflicted with diarrhea showed that the litters born before and after clean boxes were supplied were not differentially susceptible to diarrhea. 3. Correlations between the incidence of diarrhea and age of parents, and seriation of litters and size of litters, showed that each of these had some influence on the numbers of litters showing symptoms of diarrhea. Litters from young parents having early parities and large litters showed highest incidences of the disease. The physiological condition of the parents common to these three factors seems to be an important consideration. 4. A higher incidence of the disease was observed in December than in either October or November. Part of the increased incidence in December was due to a greater number of large litters, early parities and younger parents. Superimposed upon physiological condition of the parents were other factors which were tentatively called seasonal influences.

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Effect of Butazolidin^{R}* upon the Fate of I^{131} in the Rat. (20049)

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During the course of the evaluation of other drugs upon the thyroidal accumulation of iodine in patients, it was observed that a patient being given butazolidin^{R} had a very low I^{131} thyroid uptake. In this study rats were used in order to measure more completely the effect of this drug upon iodine metabolism.

Methods. Young albino rats were given butazolidine^{R} by subcutaneous injection at dosage levels ranging from 8 to 200 mg/kg body weight. In some groups of animals this was followed immediately by a tracer dose of 5 μc of I^{131} intraperitoneally. No additional iodide was added in these studies; thus the administration of the I^{131} served only to label the iodide pool of the body. The total amount of iodide present in 5 μc of I^{131} is calculated to be less than 1 x 10^{-3} μg. As will be noted in Table I, the uptake of I^{131} by control animals varied as much as 100%. Although the experimental and control animals for each group are of the same lot and origin, both Sprague Dawley and Slonaker strains of rats were employed in the several experiments combined in Table I. In our hands, Sprague Dawley rats recently received from Wisconsin have much higher thyroid uptake of I^{131} than that observed in rats bred locally. We believe that this merely represents a smaller iodide pool in rats imported from the Midwest since this higher uptake is reduced to that seen in local rats after a period of several weeks in their new environment. These animals were sacrificed 20 hours following I^{131} administration. Other groups of rats were given the drug daily for periods of 10 to 16 days in which the last injection of the drug was followed by 5 μc of I^{131}. After sacrifice selected organs of all of the rats were measured for I^{131} with a scintillation counter in which the error in measurement was less than ±4%. The geometry of the scintillation counter was such that 8% of all of the γ disintegrations occurring in the sample was observed as counts. The beta particles associated with I^{131} decay were completely absorbed with a 200 mg cm^{-2} aluminum filter and were not seen by the counter. Because of this, the wet tissues were counted directly for I^{131} by placing them in metal dishes 5 cm from the crystal of the counter. Corrections for mass absorption or small differences in geometry were not necessary when the samples were counted in this manner. The tissues of animals which were given butazolidine^{R} for longer periods of time were also examined for pathological changes and compared to the controls.

Results. The data as summarized in Table I show that the administration of butazolidine^{R} once to rats will reduce the amount of a test dose of I^{131} accumulated by the thyroid. The degree of reduction is directly related to the quantity of the drug given. More of the I^{131} given was excreted in the urine of the animals receiving the drug than by the controls. This amounted to 167, 171, 191, 400% of normal for the 1, 4, 10 and 20 mg dose levels respectively. Relatively less of the I^{131} tag was observed in plasma, kidney, GI tract plus contents, liver and skin in the group receiving 20 mg of the drug and it was observed to be about 50% of normal as shown in Table II.

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* Butazolidin^{R} is 3,5-dioxo-1,2-diphenyl-4-n butylpyrazolidin. Butazolidin^{R} was synthesized and supplied by Geigy Co., New York.

TABLE I. % of I^{131} Taken Up by Thyroid in Control Rats and in Rats Receiving Amounts of Butazolidin^R Shown Below. 5 rats in each group.

	mg to each rat				
	1	4	10	20	40
% in thyroid	10 ± 2.6*	5.4 ± 1.6	1.5 ± .1	1.8 ± .4	.33 ± .02
Avg body wt	119	126	119	207	190
	Control rats for each group				
% in thyroid	42 ± 1.7	42 ± 1.7	42 ± 1.7	44 ± 3.2	21.4 ± .22
Avg body wt	120	120	120	201	188
Origin of rat	S.D.†	S.D.	S.D.	S.D.	Slonaker

* Stand. mean error $= \pm \sqrt{\frac{\Sigma \text{dev}^2}{n(n-1)}}$.

† Sprague Dawley.

TABLE II. Comparison of Relative I^{131} Concentration in Tissues of Rats Receiving One Dose Butazolidine^R and 10 Doses of the Drug over a 10 Day Period. Animals sacrificed 24 hr after I.P. administration of I^{131}. 5 rats in each group.

	% of normal tissue uptake	
Tissue	20 mg butazolidin^R once, %	2 mg butazolidin^R daily, 10 days, %
Plasma	46	140
Kidney	52	133
G.I. tract	41	160
Liver	50	129
Skin	60	134

The balance of the rat which was composed of muscle primarily contained as much of the I^{131} tag as the controls.

When rats were given 2 mg of the drug daily for a period of 10 days and in another case 5 mg daily for 16 days, the thyroid uptake of I^{131} returned to almost normal limits. For example, in the former group the controls took up 22.2 ± 2.2% and the treated rats 19.4 ± 3.1%. In the latter group receiving 5 mg of the drug daily, the controls took up 23 ± 1.7% as compared to an uptake of 23 ± 2.6% for the treated animals.

The administration of butazolidin^R for a period of 10 days resulted in an increase of the I^{131} tag in the tissues of the rats which was observed to be 30 to 60% greater than the controls and in contrast to the lower tissue concentration of I^{131} observed when the drug was administered once (Table II).

An examination of the thyroid, bone marrow, liver, lungs, heart, spleen, muscle, ureter, adrenals, retroperitoneal fat, spinal cord, thymus, and pancreas for histological changes revealed no changes which could be attributed specifically to the butazolidin administered.

A one dimensional water chromatogram using distilled water with increasing amounts of iodide plus I^{131} suggests that butazolidin^R is capable of combining directly with iodine as iodide (Fig. 1). The mode of action of the drug in preventing iodide accumulation by the thyroid appears to be of the nature of a competition for iodide in the body.

Discussion. These data suggest that the drug butazolidin^R can alter the manner in which iodide is normally handled in the body. *In vitro* studies with chromatograms suggest that this presumably is due to the ability of the drug to combine with iodide in the body. By

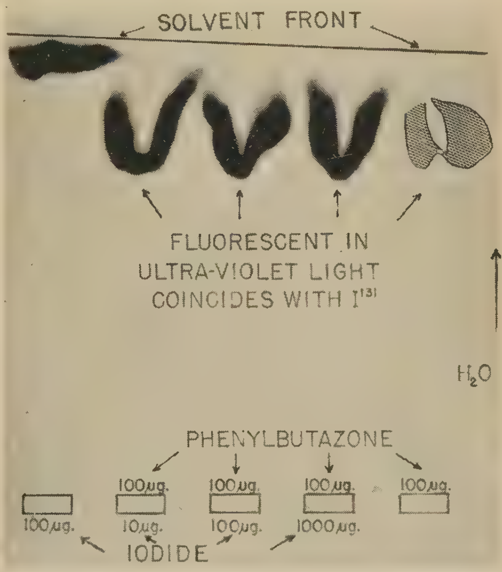

FIG. 1. A chromatogram of iodide and butazolidin^R plus iodide using I^{131} as a tracer and water as a solvent.

virtue of this capture, thyroidal accumulation of iodide and tissue content of iodide is reduced to a level much lower than normal. When the drug is administered daily, "a steady state" of drug concentration is achieved in the body. The presence of the drug in the tissues of the body increases their iodine concentration to values which are greater than normal. When the iodine affinity of the drug remaining in the body is satisfied, then thyroidal accumulation of iodine returns to normal levels.

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Diffusibility of Dyes in Buffered and Unbuffered Solutions.* (20050)

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In aqueous solutions the rate of diffusion of a substance depends primarily on its concentration gradient. In biological fluids the presence of buffers and other cell constituents may have a significant influence on the rate of diffusion. The purpose of this investigation was to compare the diffusion of basic dyes in aqueous medium, and in acid buffer. The acetate buffer was chosen because most dyes are relatively soluble in this buffer.

Method. The two half-cells were made from centrifuge tubes. Dimensions: 6.0 cm high and 2.5 cm in diameter with a side arm 1.0 cm in diameter and 1.0 cm long at a distance of 1.0 cm from the bottom of the main tube. The cellophane membrane (Eimer and Amend 8-667) was clamped between 2 rubber stoppers of 1.0 cm thickness with a bore of 0.9 cm. The side arms of the half-cells were then inserted into the rubber stoppers. Each half-cell contained either 15 ml of dye (1×10^{-4} M) in acetate buffer (pH 5.0) or water. The intensity of the diffused dyes was measured at appropriate intervals in a Beckman Spectrophotometer (Model DU). For each dye there was determined a standard concentration curve in order to transform the observed optical densities into micrograms. The *cell constant* was determined by allowing a 0.1 N HCl solution to diffuse into water. The diffused acid was measured with a Beckman pH-meter, or titrated with 0.01 N NaOH using phenolphthalein as an indicator. Ohölm(1) found that the diffusion coefficient "D" for 0.1 N HCl at 5° = 1.85 cm^2/day. The cell constant $K = D \times t/Q$. Q equals the number of ml of diffused acid expressed in units of the original concentration (the latter having a unit value of 1), and t equals time. It was found that 0.238 ml of 0.1 N HCl diffused during 30 minutes. Therefore, $K = 1.85 \times 0.0208/0.238 = 0.162$. K represents the ratio h/A, h is the effective distance through which diffusion takes place. A is the effective area of the surface available for diffusion. The diffusion coefficient $D = K \times Q/t$. The values for D are given in Tables I and II, t = 1 day. Q = micromoles (1×10^{-6} M) of diffused dye in 24 hours. In the first group of experiments the dyes were dissolved in water and allowed to diffuse into water. In the second group, the dyes were dissolved in acetate buffer (pH 5.0) and allowed to diffuse into the same buffer. In the third group there were four sections, Table III: a) The buffer in the half-cell (A) which contained the dye was diluted while that in half-cell (B) remained constant; b) the buffer in both half-cells was diluted simultaneously; c) the buffer in half-cell (A) was diluted with 10% water; and d) the concentration of the dye was varied in both water and buffer. The results are reproducible within 2-5% depending on the tinctorial power of the dye.

Results. In Table I we find the dyes arranged in decreasing order of their diffusibility "D" in water. "Stomach/blood" is the ratio of the concentration of the dye found in the

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TABLE I. Diffusion in Water through a Cellophane Membrane.

Compound conc., $1 \times 10^{-4}M$	Color index	Diffusion coeff.*	Stomach/ blood(2)	Membrane delay (hr)	pH
Thionin	920	.60	36.8	.75	5.4
Pyronine B	741	.43	5.7	.5	4.55
Acridine red	740	.34	8.4	.3	4.9
Safranin O	841	.29	27.3	2.4	6.02
Methylene blue	922	.27	15.6	.5	5.76
Methylene green	924	.26	13.6	2.6	5.7
Rhodamine B	749	.23	6.1		4.38
Toluidine blue	925	.21	36.4	2	5.02
Brill. cresyl blue	877	.18	11.3		4.27
Chrysoidin Y	20	.13	75	.5	4.9
Neutral violet	826	.13	27.2	.5	5.5
Bismarck brown	331	.12	26.5	6.8	4.1
Neutral red	825	.07	26.4	1.6	5.5

* cm^2/day.

TABLE II. Diffusion in Acetate Buffer (pH 5)* through a Cellophane Membrane.

Compound conc., $1 \times 10^{-4}M$	Color index	Diffusion coeff.†	Stomach/ blood(2)	Membrane delay (hr)
Methylene blue	922	.48	15.6	
Methylene green	924	.42	13.6	
Neutral red	825	.39	26.4	.8
Safranin O	841	.36	26.3	
Neutral violet	826	.36	27.2	
Brill. cresyl blue	877	.35	11.3	
Pyronine B	741	.33	5.7	
Chrysoidin Y	20	.29	75	
Acridine red	740	.24	8.4	
Bismarck brown	331	.24	26.5	3.4
Thionin	920	.22	36.8	
Rhodamine B	749	.21	6.1	
Toluidine blue	925	.18	36.4	

* The buffer was prepared by mixing 590 ml of .2M acetic acid with 141 ml of .2M sodium acetate (3).

† cm^2/day.

stomach to the concentration in the blood as determined by Ingraham and Visscher (2). In column 5 is listed the lag in diffusion caused by the membrane. We see that it takes different times for the membrane pores to become saturated with the dyes. In column 6 the pH values of the aqueous solutions are given. There seems to be no obvious relation between diffusibility and the pH of the solution.

In Table II the dyes are arranged in decreasing order of their diffusibility in acetate buffer (pH 5.0). Most of the dyes diffuse faster in buffer than in water. However, Acridine red, Rhodamine B, Pyronine B, Toluidine blue and Thionin diffuse faster in water than in buffer. It invariably takes more time for the membrane to become saturated with the dye in water than in buffer. Whereas a lag in diffusion by the membrane is observed for most dyes in aqueous medium, in acetate buffer only Bismarck Brown and Neutral Red are delayed. Their delay in buffer is only 44% and 50% respectively of the values in water. The spread between the fastest diffusing compound and the slowest is greater in water than in buffer, the comparative ratios being 8.6 and 2.7. Thionin is the fastest in water but one of the slowest in buffer. The probable explanation of these differences involves both hydration (or hydrolysis) and

TABLE III. Effect of Dilution of Buffer on Rate of Diffusion.

Half-cell A, dye + % buffer*	Half-cell B, % buffer	Diffusion† Methylene blue	Diffusion† Methylene green
a. 100	100	.48	.42
75	100	.82	.77
50	100	1.02	.89
25	100	1.28	1.18
0	100	1.51	1.51
b. 100	100	.48	.42
75	75	.43	.39
50	50	.42	.36
25	25	.32	.36
0	0	.27	.26
		Acridine red	Pyronine B
c. 100	100	.24	.33
90	100	.36	.50
		Methylene blue	
d.	Conc.	D (H_2O)	D (buffer)
	$.5 \times 10^{-4}M$	.15	.48
	$1 \times 10^{-4}M$	.27	.48

* Conc. of 100% buffer, .2M; 75% buffer, .15M; 50% buffer, .1M; 25% buffer, .05M; 90% buffer, .18M.

† cm^2/day.

ionization. Those substances that are highly ionized and which do not react with water should diffuse better in water. In buffer their ionization is repressed and diffusion is slower. On the other hand, if a substance is readily hydrated it might be expected to diffuse more slowly in water. A third possibility stems from the fact that in water the amines are in the form of their hydrochlorides while in acetate buffer they are, for all practical purposes, acetates. Certain acetates are known to dissociate to a lesser extent than the corresponding chlorides. In such cases an inhibition of diffusion would occur in buffer. Thionin, Toluidine Blue and Pyronine B are among the stronger bases(4) so that the increased ionization in water is a dilution phenomenon. Rhodamine B and Acridine Red, on the other hand, are weak bases and hydrolysis should be the predominant factor. That it is not, seems to point to the greatly reduced dissociation of their acetates. Probably the most important finding from the biological viewpoint is that the *order of diffusibility* is different in acid buffer than in water, Tables I and II. There seems to be little relation between the rates of diffusion and the ability of the stomach to secrete the dyes. The influence of dilution was studied for Methylene Blue, Methylene Green, Acridine Red and Pyronine B, Table III. The observed results show that the rate of diffusion depends not only upon the concentration gradient (Fick's Law) but also upon the concentration of buffer present in both half-cells.

If solutions of Methylene Blue and Methylene Green (1 x 10^{-4} M) are compared and only the buffer in the dye compartment (Half-cell A) is diluted by 25%, 50%, and 75% respectively and allowed to diffuse into the full strength buffer (Half-cell B), one notices that the diffusion increases almost linearly, Table III, a. However, if both half-cells A and B are diluted by 25%, 50%, and 75%, there is little effect, except possibly for the 25% buffer with Methylene Blue, until pure water is reached. The pH is unchanged by dilution.

Since dilution increases the ionization of the dye in half-cell A, the rate of diffusion should be increased. Now, if half-cell B contains the concentrated buffer it represses the ionization and thus decreases the back diffusion. If, however, half-cell B is diluted in the same proportion as half-cell A, it no longer can repress the ionization and the dye diffuses back into half-cell A. The ultimate result is a decrease in diffusion rate because of increased hydration. The latter effect is less striking than the former.

Acridine Red and Pyronine B are difficultly soluble, and their diffusion was studied in full strength buffer (Half-cell A) and then compared with the diffusion after diluting half-cell A by 10% water and allowing to diffuse into full strength buffer (Half-cell B), Table III, c. Acridine Red showed in 10% dilution an increase in concentration of 6.35%, whereas the rate of diffusion was increased by 50%. In Pyronine B the concentration is increased by 9.1%, whereas the rate of diffusion is increased by 50%. Even 10% dilution thus shows the same effect as was observed for Methylene Blue and Methylene Green, Table III, a.

We also noticed that the membrane has a different capacity for the same dye in different media, Table III, d. Methylene Blue in water for a concentration of 0.5 x 10^{-4} M has a value of D = .15. If we double the concentration the value for D increases to .27. The same dye in acetate buffer does not change its value of D = .48 on doubling its concentration. One would be apt to conclude that .48 represented the capacity of the membrane, but Table III, part a, shows that diffusion values above 1.5 may be obtained with the same membrane by using a different concentration in each half-cell.

Summary. The rate of diffusion of basic dyes depends not only on the concentration gradient but also the medium. The diffusion of dyes into a concentrated buffer is faster than into a diluted one. The concentrated buffer represses the ionization, and thus retards the back diffusion. If the buffer compartment is diluted in the same proportion as the dye compartment, the rate of diffusion of the dyes is only slightly affected, if at all, by a 4-fold dilution. In diffusion studies with Methylene Blue it was found that the membrane has, in different media, a different diffusion capacity. This work suggests that conclusions based on diffusion measurements made on simple aqueous solutions may not be equally valid for biological systems.

The author wishes to thank Dr. F. E. Ray for his suggestions and criticisms.

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Effect of Butazoldin^R on I^{131} Uptake by the Thyroid Gland—II. (20051)

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(Introduced by K. G. Scott.)

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Butazolidin^R* is a pyrazolon derivative which has been found to be useful in the treatment of various rheumatic diseases. In the course of a clinical investigation of effectiveness of this compound a radioactive iodine uptake test for thyroid function was performed and uptake measured 6% at 24 hours, about one-third of normal average and inconsistent with the patients' appearance. This led to the studies reported here.

Methods. The I^{131} uptake of the thyroid gland was determined prior to and after the administration of butazolidin^R for 2 or 3 days. Each of eleven euthyroid patients and one hyperthyroid patient was given 5-15 μc of I^{131} and after 24 and 48 hours, the radioactivity over the thyroid gland, expressed as percent of the administered dose, was measured using a scintillation counter. After the last measurement a daily dose of 1.0 g of butazolidin^R was given intramuscularly to each of the patients for 2 or 3 days. A second I^{131} uptake test, using 30-50 μc was performed on 4 of the euthyroid patients 24 hours after the last of 2 daily injections. The remaining 8 patients, including the hyperthyroid case, were tested concomitantly with the administration of the third daily injection of butazolidin^R. Studies in this laboratory have shown, as might be expected, that the thyroid uptake of a dose of I^{131} is not related to the amount given in μc. The number of iodine atoms which are present in a test or therapy dose of I^{131} represents a quantity which is less than 0.1 μg of iodine.

TABLE I. % I^{131} Uptake by Thyroid at 24 Hr in 14 Patients Receiving Repeat but Variable Amounts Expressed in Microcuries.

Patient	Diagnosis	Dose I^{131} (orally), μc	Date	% uptake
L.	Hyperthyroid	15	8/16/52	66
		125	17	68
		3000	19	65
J.	”	25	9/14/51	65
		6700	20	68
C.	”	66	3/27/52	66
		9100	31	64
W.	”	9	8/ 8/52	75
		8800	13	76
R.W.	”	100	5/ 9/51	52
		7500	12	53
Q.W.	”	120	5/ 1/52	67
		10000	9	64
C.W.	”	20	10/27/51	60
		1000	11/ 1	65
”	”	100	9/27/52	73
		4530	10/ 3	73
K.	Cardiac	15	4/20/52	44
		10000	5/26	45
B.	Euthyroid	46	11/ 4/52	24
		100	12/16	21
V.W.	Thyroid block	5	12/ 3/52	1–2
		50	4	1–2
G.B.	Euthyroid	5	5/28/52	10
		140	6/24	11
H.	”	2	4/16/52	50
		30	17	48
G.W.	”	2	4/16/52	20
		30	17	18

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* Butazolidin^R is 3,5-dioxo-1,2-diphenyl-4n-butyl-pyrazolidin and is marketed as *Butazolidin*^{R}. We wish to thank the Geigy Co. for a generous supply of this drug.

TABLE II. Effect of Butazolidin^R on I^{131} Uptake by Thyroid Gland in 11 Euthyroid Patients and 1 Hyperthyroid Patient.

		% uptake by thyroid 24 and 48 hr following oral admin. of I^{131}				% reduction in I^{131} uptake after butazolidin^R admin.	
		Pre butazolidin^R		Post butazolidin^R			
Patient	Diagnosis	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr
W.B.	Gout	31	32	4	4	87	88
B.	”	23	24	5	4	78	83
S.	”	21	21	2	2	90	90
F.	”	15	18	5	5	67	73
J.*	Psoriasis	26	35	11	18	58	49
H.	Cerebral arterio-sclerosis	13	15	1	2	93	87
E.B.	Chronic hepatitis	17	21	4	7	77	67
H.S.†	Lymphocytic leukemia	18	21	7	7	61	67
T.	Tbc. adenitis	28	26	4	5	85	81
S.W.	Pulmonary density (?)	8	8	1	1	88	88
W.	Rheumatoid arthritis	10	11	1	1	90	89
D.	Hyperthyroidism	57	54	12	8	80	85

* Patients 1-4 received daily 1 g I.M. inj. of butazolidin^R for 2 days; oral I^{131} given 24 hr after last dose of butazolidin^R.

† Patients 5-12 received daily 1 g I.M. inj. of butazolidin^R for 3 days; oral I^{131} given concomitantly with last dose of butazolidin^R.

As shown in Table I, uptake is not dependent upon the dosage of I^{131} employed.

Results. The data in Table II show in all cases a reduction in the uptake of I^{131} by the thyroid gland following daily intramuscular injections of butazolidin^R for 2 or 3 days. In more than half the cases the drug reduced the uptake to less than 20% of the previous level. In all but one of the comparisons (24 and 48 hours) the uptake after butazolidin^R is less than half what it was before it. The characteristic effect of butazolidin^R on the thyroid uptake of I^{131} of 3 of the euthyroid patients (Table I, patients 1,2,3) is shown in Fig. 1.

The thyroid I^{131} uptakes in the patient with hyperthyroidism before and after the drug are shown in Fig. 2. The rather high level, 57%, was brought down to a low euthyroid level, 12% by 3 g of butazolidin^R.

Comment. In 12 patients (one hyper-

Effect of Phenylbutazone on I^{131} Uptake by Thyroid Gland in Three Patients.

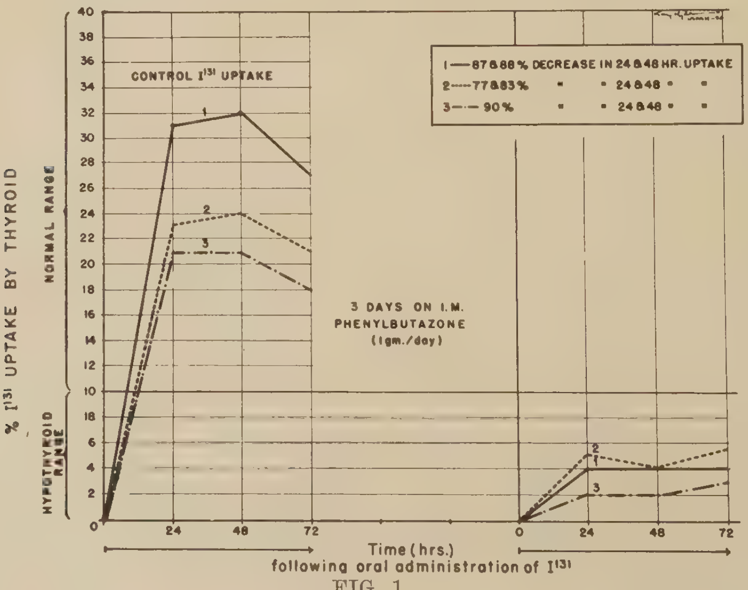

FIG. 1.

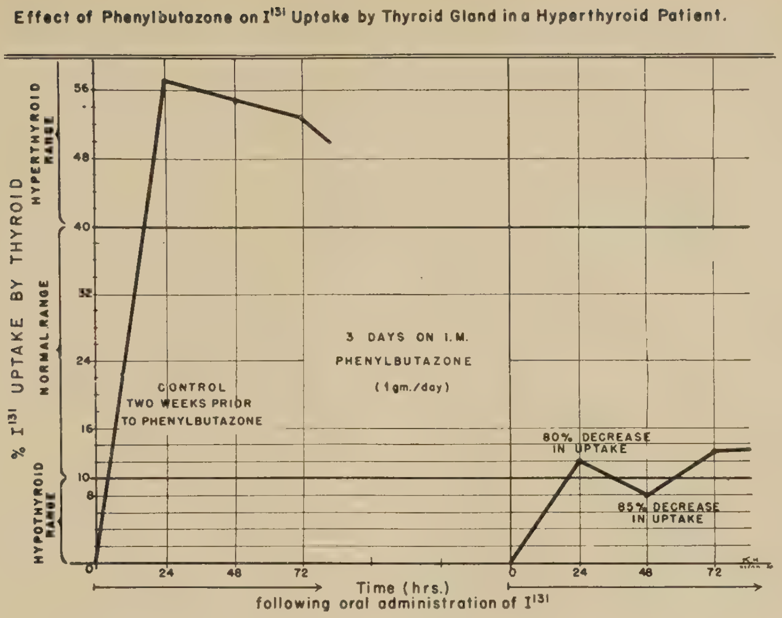

FIG. 2.

thyroid) butazolidin® interfered with uptake of I^{131} in the thyroid gland. The extent of this action is now being observed through a series of I^{131} uptakes during the continuous prolonged oral administration of butazolidin®.

At the present time it may be stated that no instance of clinical hypothyroidism has been observed in our group of gout and arthritic patients, some of whom have been treated with the drug for as long as 12 months.

The mode of action of this compound on the thyroid was unknown at the time of these observations; however, possible mechanisms are suggested in another publication.†

Summary. The intramuscular injection of butazolidin® in a daily dose of 1.0 g for 2-3 days reduces I^{131} uptake by the thyroid gland.

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† Effect of Butazolidin® upon the Fate of I^{131} in the Rat-I, Scott, Kenneth G., Frerichs, John B., and Reilly, William A.

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Effect of Pooling of Liver Slices upon Protein Synthesis in Guinea Pigs.

(20052)

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Assuming the radiation emitted by S^{35}-methionine incorporated *in vitro* into liver protein to be x counts/min/unit in one animal, and y counts/min/unit in another, if the 2 livers were pooled, in equal units, a value of $x + y/2$ counts/min/unit would be expected. In the course of work involving guinea pigs, it was noted that pooled liver slices did not incorporate S^{35}-methionine at the rate expected from the samples singly; hence the following study was made. As will be shown, pooled values were consistently lower than theoretical values.

Materials and methods. Female guinea pigs of mixed breeds and colors, weighing 350-600 g, were used. Water and standard rabbit ration (Red Chain) were supplied *ad lib.*, and fresh vegetables were given 5 days per week. The guinea pigs were killed by a blow to the head, and the livers immediately removed and placed on ice. Slices were made free-hand with razor blades and placed in cold buffer of the following composition per 100 ml:

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* Captain, Medical Corps, A.U.S.

70.2 ml	.154 M	NaCl
1.4	.11	CaCl
1.4	.154	KCl
27	M/10 phosphate pH 7.25	
200	mg glucose	

After the liver was sliced, the buffer was absorbed with gauze, and the slices weighed. Ten g of slices and 10 ml of buffer were placed in a 125 ml Erlenmeyer flask. S^{35} labeled L-methionine† was added, so that a concentration of about one microcurie/ml was present. The amount of methionine varied from 0.35 to 3.0 mg, but was constant for a given experiment. The time from death of the animal to placing the slices on the bath was about 90 minutes in all cases.

TABLE I. Error of the Method.

Exp. No.	Guinea pig No.	CPM/ 4.49 cm²	Variation, %
2	1	3840	7.3
	Duplicate	3560	
3	2	3590	8.2
	Duplicate	3910	
4	3	4560	4.6
	Duplicate	4570	
	Triplicate	4360	

Identical flasks were set up for 3 control animals.

TABLE II. Effect of Pooling on Guinea Pig Liver Protein Synthesis.

Exp. No.	A	B	½(A+B)	C	$\frac{C}{½(A+B)} \times 100$
5	6250	4940	5600	3260	58
6	2690	3860	3270	1990	61
7	4850	3390	4130	2560	62
8	8230	3970	6100	3870	63
9	3350	3400	3380	2180	64
10	6370	4280	5320	3440	64
11	3730	3450	3570	2380	66

Values are counts/minutes/4.49 cm².
A = value from first animal.
B = " " second " .
C = " " equal parts of first and second animals.

A typical experiment. Two female guinea pigs, A and B, weighing 405 and 384 g respectively, were killed and the livers sliced. Flask 1 contained 10 g of liver from A and 9.65 ml of buffer. Flask 2 contained 10 g of liver from B and 9.65 ml of buffer. Flask 3 contained 5 g of liver from A, 5 g from B, and 9.65 ml of buffer. To each of the 3 flasks was added 0.35 ml of solution containing 19.8 μc S^{35} and 1.6 mg of L-methionine. The flasks were placed in a Warburg bath at 37°C, allowed to equilibrate, and stoppered. They were shaken for 2 hours and then opened for 5 minutes to permit renewal of air. After a total of 4 hours, the flasks were removed from the bath and the contents immediately homogenized in a cold room at 3°C. The liver protein was precipitated by the addition of an equal volume of 10% trichloroacetic acid. The time from removal from the bath until the protein was precipitated was 15 minutes. The precipitates were washed three times with 0.05 M non-radioactive DL-methionine, and once with water. The residues were spread on porcelain Petri dishes and dried overnight under a 60-watt lamp. The dried material was ground in a mortar and 300 to 600 mg were placed in planchets of 4.49 cm² area. The surfaces were tamped level. Specimens were set up in duplicate. Each sample was counted twice in a Nucleometer,‡ at infinite thickness for a total of 12,800 counts each. Infinite thickness was determined to be <200 mg/4.49 cm². All raw counts were >10 x background. The mean variation in counts between the 41 pairs of duplicates set up in separate planchets was 3.4%. To determine *adsorption of S^{35}-methionine*, the procedure was carried out as above at zero time after mixing. The recorded experimental figures have been corrected for this adsorption. All counts were normalized to the basis of 1 μc S^{35}/ml of the flask contents. All counts are expressed as counts per minute/4.49 cm².

Results. Adsorbed methionine. In Exp. 1, performed at zero time, the first guinea

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† Purchased from Abbott Laboratories, N. Chicago, Ill.

‡ Internal gas flow radiation counting meter, manufactured by Radiation Counter Laboratories, Skokie, Ill.

TABLE III. Effect of Heat and Supernate upon Pooling Effects.

Exp. No.	Ingredients	A	B	½(A+B)	C	$\frac{C}{½(A+B)} \times 100$
12	Normal slices (A) + heated slices (B)	9120	0	4560	5610	123
13	Normal slices (A) + normal + supernate (C)	7090			11380	*160
14	Supernate added to pooled sample (C)	4190	11580	7890	5150	65

Values are counts/minutes/4.49 cm^2.

* In this case, the expected value for C is the same as A.

pig adsorbed 200 CPM, the second 230 CPM, and the pooled sample 213 CPM. The round figure of 200 CPM was used as the adsorption correction.

Error of method. The reproducibility of the method is shown in Table I. In these 3 experiments, duplicates or triplicates were set up and run as described. The overall error of the method appears to be around 8%.

Effect of pooling. Table II represents the results in pooled livers. Although there was a 3-fold variation between the lowest and highest uptake, the percent of the theoretical pooled uptake was amazingly constant, being between 58 and 66% of the theoretical 100%. The mean uptake of the unpooled specimens was 4480 CPM. For convenience, the experiments are arranged with percentages in ascending order, although this was not the experimental sequence.

Effect of heating and supernate. Table III demonstrates the effect of heat and supernate on S^{35} uptake. In Exp. 12, one liver was heated to 80°C for 60 minutes on a water bath, and the experiment then carried out as usual. A moderate stimulation was found. In experiment 13, one liver was sliced and placed on the Warburg without S^{35} methionine. After shaking 4 hours at 37°C, the slices were spun down and the supernate frozen. The following morning another guinea pig liver was sliced and one flask set up as usual. A duplicate was set up with 7 ml of buffer replaced by 7 ml of supernate prepared the preceding day. This second flask showed a marked increase over the flask with buffer alone. In Exp. 14, flasks A and B were set up as usual, but to the pooled slices in flask C was added an equal volume of supernate from a third guinea pig, prepared as described for Exp. 13. A depression to 65%, the same value found without added supernate, was found.

Discussion. No reports could be found on changes of *in vitro* metabolism incurred by the pooling of tissues. Rutman, Dempster and Tarver(1) reported variations in the uptake of S^{35}-methionine by rat liver slices to be dependent upon the strain used. Canzarelli, Rapport and Guild(2) report that the uptake of C^{14}-alanine into rat liver slices can be decreased to 17% of normal by carbohydrate deprivation for 24 hours. A combination of these factors probably accounts for the range of uptake in our animals, although they were feeding *ad lib.* However, this has no obvious relevance to the "pooling effect." Since the period of contact was only 4 hours, antibodies could scarcely be expected to have formed. Furthermore, even a previously immunized animal produces little antibody in the liver(3). Hence, some genetic mechanism other than antigenic differences must be acting. The guinea pigs were not inbred, and all color combinations were used, but no differences in depression were found.

Although the substance, or substances, responsible for this depression must go from one set of cells to the other, Exp. 12, using prepared supernate, did not confirm this concept. A stimulation, rather than a depression, was found. Such an increase could be due to the addition of substrate in the supernate, since net hydrolysis occurs in all slice experiments, and after 4 hours incubation the amino nitrogen is always increased. Exp. 13, using heated tissue, would also release substrates, and it, too was stimulatory. Despite these stimulatory effects, Exp. 14 demonstrated that the addition of such substrates will not alter a depressing effect between two sets of different but viable cells. Much more work is needed to elucidate the mechanisms involved in these observations.

Summary. 1. The uptake of S^{35}-L-methionine into the protein of pooled and non-pooled liver slices in guinea pigs has been studied. 2. Defining the mean of liver uptakes from 2 animals as 100%, when the two guinea pig livers were pooled, an uptake of 58 to 66% was found. 3. No evidence for a diffusible inhibitor could be demonstrated.

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Changes in Eosinophil Count of Mice with Venisections* Repeated at Intervals of Several Days.† (20053)

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In the course of studies on characteristic eosinophil levels(1) it was noted that the mean eosinophil count of groups of inbred mice obtained at four-day intervals showed marked differences from one sampling time to the other. Such results were obtained despite the observance of certain precautions to control the physiological eosinophil rhythm(2). These included 1) not only the procedure of sampling at the same times of day, but 2) the equally relevant standardization of experimental animals by means of a regimen of single housing for at least nine days in an air conditioned room at the temperature of 78 ± 2°F with standardized lighting (light from 06:00 to 18:00 and darkness from 18:00 to 06:00)‡ and *ad libitum* feeding.

The methods of handling and counting have been described(1). The counts were made at the times indicated in the tables. The periods chosen are during the rising phase of the usual daily rhythm. It is noted that 2 counts were obtained on each of the days of observation at an interval of 4 hours. The first of the 2 counts for each animal and for each day of observation is presented.

A summary of the data on eosinophil counts obtained under these circumstances is presented in Table I. It is apparent that the mean count obtained for the third venisection is lower than that obtained for the first venisection for each of the 10 stocks of mice investigated.§ It appeared undesirable to accept these observations as representative of a more general phenomenon in mice, since the data were obtained from very homogeneous populations of animals. It does not follow *a priori* that results valid for particular stocks and circumstances would necessarily be valid for other biological material, even though closely related. Yet it appears that they are valid.

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* Although the term venisection is employed for linguistic convenience it is recognized that the samples of blood obtained from a small incision in the mouse's tail were either venous or arterial, or mixed. For the purposes of this investigation it is believed that the distinction between venous and arterial blood is not significant.

† This investigation was supported by a research grant from the National Heart Institute of the National Institutes of Health, U. S. Public Health Service.

‡ Standardized environmental circumstances, especially controlled lighting, appear to be necessary to assure constancy of the temporal placement of physiological eosinopenia within the 24-hour period (3).

§ These mice have been described(1).

TABLE I. Mean Eosinophil Levels in Three Successive Determinations at 4-Day Intervals on Several Stocks of Mice.

Stock	No. of animals	Eosinophils/mm^3 of tail blood* Median	Mean
B_1	17	969	1022
	14	825	1022
	"	750	684
B_4	15	687	700
	14	675	661
	"	594	580
D_8	"	259	297
	"	212	195
	"	119	142
ZD_8F_1	"	125	130
	"	131	129
	"	87	83
Z	"	181	179
	"	150	176
	"	106	119
Z_B	"	169	198
	15	106	113
	"	75	91
A	14	175	166
	"	75	112
	"	75	85
A_X	"	162	168
	"	100	100
	"	87	81
$Z_BA_XF_1$	11	131	143
	"	100	115
	"	112	125
$A_XZ_BF_1$	10	112	129
	"	119	113
	"	94	110

* Counts obtained from 06:00 to 10:00.

Comparable data from a larger group of genetically less homogeneous mice are presented in Table II. Individual counts are listed to illustrate the variability of the changes in individual animals. The general trend, however, is again a slight but progressive decrease in number of eosinophils from one sampling time to the other.

Table III summarizes the results of an analysis of variance applied to these data. In the presence of the expected significant inter-individual differences the difference in mean eosinophil level between the successive experiments is also significant below the one per cent level (the F value required for the 1 per cent level is 3.48; that obtained for the differences between successive experiments is 7.24). Whatever may be the cause of these differences, it appears obvious that the changes are not simply intra-individual random variations. Rather the past history of a mouse—including venisections performed several days prior to an eosinophil count—has a definite effect upon the number of circulating eosinophils.

Two approaches are practicable for control of factors like these venisections in the experimental analysis of the effects of a given

TABLE II. Five Successive Eosinophil Counts on 2-Month-Old Male ZBC* Mice, 44 Mice.

	Eosinophils/mm^3 tail blood—successive determinations† 1	2	3	4	5
	42	39	36	22	17
	36	28	45	58	45
	55	78	62	73	50
	87	100	139	111	83
	226	139	92	92	75
	126	97	51	125	42
	55	47	222	239	76
	396	81	67	41	39
	207	246	37	70	303
	101	62	133	55	81
	83	129	98	84	69
	106	147	76	58	58
	181	172	89	83	129
	80	128	92	33	47
	87	69	78	42	44
	69	53	33	36	22
	70	69	28	47	44
	61	72	105	20	28
	27	66	117	51	42
	28	92	83	64	37
	133	158	72	84	95
	204	167	23	41	33
	87	103	87	34	28
	123	34	22	20	33
	23	42	103	9	17
	148	142	109	114	129
	262	193	220	195	80
	117	140	37	78	80
	53	204	173	172	151
	279	47	254	133	181
	94	86	150	120	30
	103	66	105	34	31
	133	289	98	67	78
	19	58	183	73	41
	285	168	81	62	62
	268	129	105	98	53
	198	162	140	66	56
	256	129	92	90	45
	31	87	61	62	90
	39	76	159	198	144
	173	222	184	158	131
	75	55	44	22	58
	39	53	105	112	28
	253	217	131	125	165
Exp. mean	125	112	101	81	72

* The fostered A and Z stocks are mated to produce either the $A_XZ_BF_1$ or the $Z_BA_XF_1$ hybrids. The progeny of F_1 females backcrossed to Z_B males is referred to as ZBC.

† Counts obtained from 02:00 to 06:00 at 4-day intervals.

TABLE III. Summary of Analysis of Variance.

Source of variation	D.F.	Variance	F	F.05	F.01
Between mice	43	99122.62	3.13	<1.50	<1.76
Between exp.	4	21104.88	7.24	<2.45	<3.48
Pooled residual	172	2914.11			

variable. An experimental design providing for all possible protection of experimental animals against extraordinary stimulation has great merits. Animals with no past history of venisection, *e.g.*, can be used whenever comparable populations of experimental animals are available. Statistically significant groups of animals can then be studied at several time points if need be. However, the other possible investigative design, involving the use of animals exposed to the same number of venisections, is often a necessary concession to practicability. It is recognized, however, that this second procedure is open to some criticism. It would approximate control of the stimulation only if mice subjected to venisection could be handled identically. And even if this were feasible it would not necessarily follow that two groups of experimental mice will react identically to venisection and/or the handling associated with this operation.

If eosinophil counts are to be dealt with as a quantitative variable, control of the past history of the animals employed will have to be established, in addition to scrupulous care for the maintenance of standardized environmental(3) and temporal(2) circumstances. It is conceivable that effects on this variable could then be evaluated as effects on a fundamental physiological cycle in mice(4) and in man(5,6).

Summary. The numbers of circulating eosinophils in the tail blood of mice have been determined at intervals of several days. A decreasing trend in mean number of eosinophils is noted with repeated venisections for various stocks of mice. The procedural implications of the finding are discussed in the light of related data. Control of the past history of experimental animals appears to be necessary if eosinophil counts are to be evaluated quantitatively.

The author is indebted to Dr. M. B. Visscher for advice, to Dr. J. J. Bittner for the mice employed and to Dr. A. E. Treloar, Division of Biostatistics, Univ. of Minnesota, for statistical help.

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Degenerative Forms of Eosinophilic Leukocytes in Lymphoid Organs.* (20054)

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Recent reports have dealt with the fate of the eosinophilic leukocyte following various stimuli which reduce their numbers in the peripheral blood stream. Padawer and Gordon (1,2) have described the process of breakdown of eosinophils in peripheral blood and in the pleural and peritoneal fluids of rats treated with epinephrine or cortisone. It has also been reported(3) that significant decreases occur in the numbers of eosinophils following their incubation *in vitro* with cortisone. The present report provides evidence that eosinophils are found normally in varying stages of breakdown in lymph nodes and that lowered

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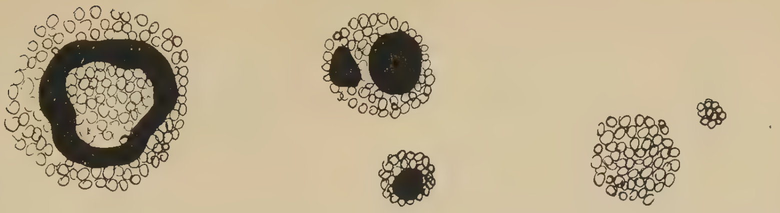
* Work done under a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.



Figures are diagrammatic representations of normal and degenerative forms of eosinophils.
FIG. 1. Normal eosinophil. FIG. 2. Eosinorrhexicytes. FIG. 3. Eosinomeres.

barometric pressures will increase the relative numbers of degenerated eosinophils in these organs.

Materials and methods. Adult male rats of a modified Long-Evans strain and weighing 265-400 g were employed. Eight rats were exposed to a simulated altitude of 25,000 ft. (282 mm Hg) for 6 hours in a specially constructed low pressure chamber. During this time they received food and water *ad libitum.* At the end of this period of exposure, they were anesthetized and decapitated. Eight untreated rats served as controls. Touch prints were made of the ileo-cecal lymph nodes. These organs were employed because they were found to contain greater numbers of eosinophils than did lymph nodes in other locations. Samples of these nodes were also fixed in Zenker-formol. Similar procedures were repeated for the spleen, thymus and other lymphoid tissues. The imprints were stained with Giemsa and the sections with hematoxylin and chromotrope 2-R(4). Peripheral eosinophil counts were made by the Randolph (5) chamber method. The evaluation of eosinophil destruction was standardized by examining the touch prints of the ileo-cecal lymph node closest to the cecum. At least 200 eosinophilic elements in this node were counted for each animal and classified as follows(6): A) Eosinophils. This includes all nucleated eosinophils possessing either a "ring form" nucleus (Fig. 1) or a solid nucleus with a chromatin network that appears normal. B) Eosinorrhexicytes. These bodies are smaller than those in the first category. The nuclear material is reduced in size and usually presents a laked appearance when stained with Giemsa. One or more nuclear fragments may be present (Fig. 2). C) Eosinomeres. These eosinophilic bodies are anuclear. They are usually spheroid and are composed of granules that are indistinguishable from those seen in the eosinophils of categories A and B. Both the size and extent of granulation of the eosinomeres are variable (Fig. 3).

Results and conclusions. Male rats weighing more than 200 g were employed in these experiments because of the greater numbers of eosinophils detected in their ileo-cecal lymph nodes than in those of smaller animals (100-180 g). Considerable numbers of eosinomeres were apparent in untreated rats and in rats exposed to the reduced pressures. They were seen in touch prints, sections, smears and in suspensions observed in a warm chamber. The size of eosinomeres varied from approximately 1 μ to 10 μ in diameter.

Examination of 5 μ sections of ileo-cecal lymph nodes often revealed the presence of eosinophils in the capsular and adjacent cortical areas. These eosinophils frequently appeared in clumps and many of them displayed marked degeneration. In the connective tissue capsule the eosinophils were spindle-shaped and arranged parallel to the capsule.

A peripheral eosinopenia was observed in the rats subjected to the lowered barometric pressures for 6 hours. In addition, a marked "shift to the right" was noted in the percentages of the 3 categories enumerated for the ileo-cecal lymph nodes (Table I). Touch prints and supravital studies have suggested that these eosinophils undergo destruction through budding and degranulation. Despite signs in touch prints of phagocytosis of lymphoid and erythroid cells by macrophages in the lymph nodes, only rarely was the ingestion of eosinophilic elements noted. Evidence of eosinophil destruction similar to that in the ileo-cecal lymph nodes has been observed also in the spleens, thymuses, and thymic, renal

TABLE I. Mean Percentages (± Stand. Errors) of Eosinophilic Forms in Ileo-cecal Lymph Nodes of Rats.

	Eosinophils	Eosinorrhexicytes	Eosinomeres
Controls (no pressure)	65.1 ± 4.8	.4 ± .2	34 ± 6.5
Experimentals (280 mm Hg 6 hr)	32.6 ± 3.2	15.3 ± 3.6	52.3 ± 4.4
P values*	<.01	<.01	<.02

* Probability values obtained from the distribution of Fisher's t. The 3 values are considered significant.

and lumbar lymph nodes. Examination of the colon, liver and lungs has revealed eosinophils of only the type A variety. Immature eosinophils have been seen occasionally in smears of spleen suspensions; however, these number fewer than 0.1% of the total eosinophils seen.

The question remains open as to whether an active process of eosinophilic leukocyte destruction occurs within the lymphoid organs or whether the degenerated eosinophilic forms represent bodies filtered out by the nodes. In addition, the mechanism for the breakdown of the eosinophil as seen in the rat exposed to lowered barometric pressures is still to be elucidated. Experiments are under way to determine the precise role played by the adrenal cortex in this phenomenon.

Summary. Degenerative forms of eosinophilic leukocytes are found commonly in ileocecal lymph nodes and in other lymphoid tissues of the rat. The relative numbers of these degenerative forms are increased in rats subjected to lowered barometric pressures.

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Influence of Small Doses of Heparin on Fat Tolerance Curves *in vivo* and *in vitro*.* (20055)

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Hahn's(1) discovery that lipemic serum could be cleared by heparin has stimulated work on the nature of the mechanisms involved. Recently, Anderson and Fawcett(2) reported that heparin added to lipemic serum *in vitro* did not clear the serum, but when a post-heparin serum sample was mixed with the lipemic serum, clearing of the lipemic serum occurred. The material present in the post-heparin serum, was called "antichylomicronemic substance." The nature of this clearing factor has been somewhat clarified by Anfinsen and co-workers(3) who isolated a serum protein (Cohn IV-I) which in the presence of heparin and a tissue factor, resulted in the formation of the "clearing factor" (Cohn III 1, 2, 3), and they were further able to demonstrate also that once formed, the complex was activated by a protein in another serum protein fraction (Cohn III-0). Block(4) and co-workers have reported that great differences exist in the degree to which sera of different

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* This study was supported in part by grant, National Heart Institute, National Institutes of Health, Bethesda, Md., and the Public Health Research Institute of the City of New York.

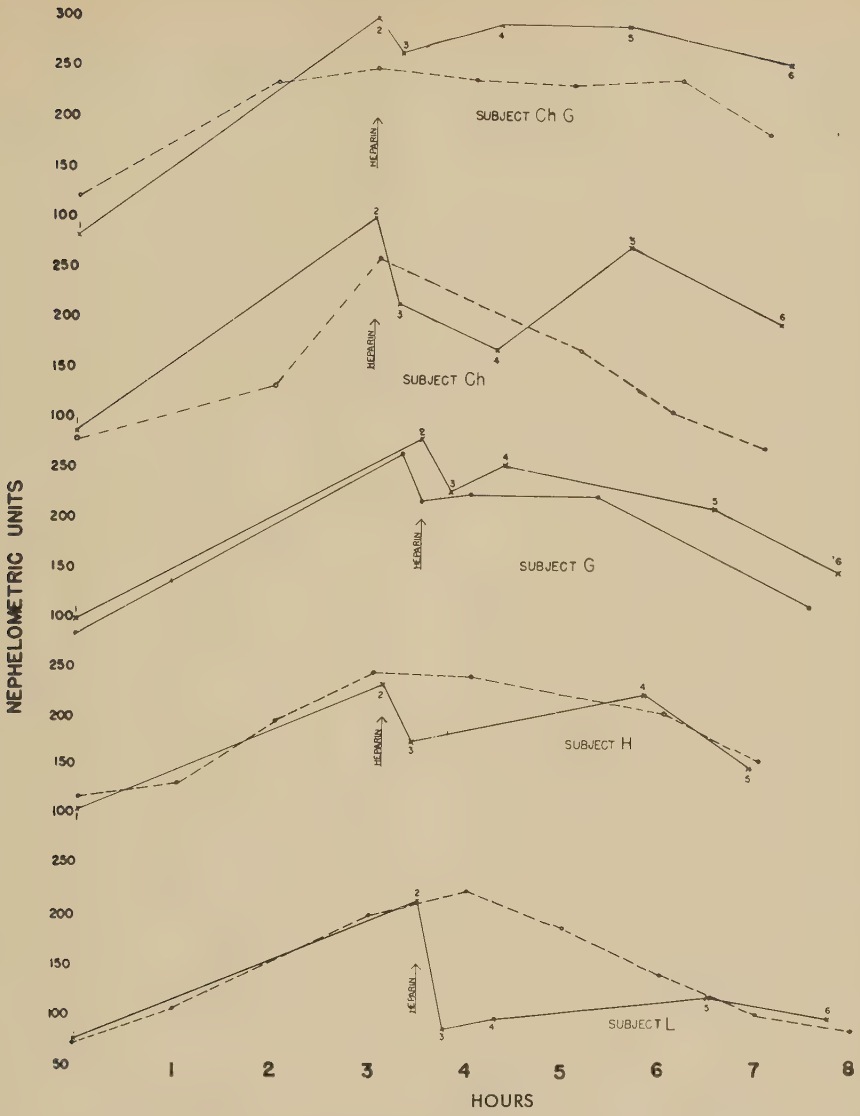

FIG. 1. The turbidity curves (×——×) of the 5 subjects who received heparin are illustrated. Except for subject G, a fat tolerance control test (○----○) was available for each subject. In the instance of subject G, 2 fat tolerance tests in which heparin was used were available (×——×, ●——●) and are presented. The numerals on each curve when used in conjunction with the subjects' initials correspond to the mixture of sera in Table I. The arrows indicate the time of heparin inj. Samples No. 4 and No. 5, subject CHG, were slightly hemolyzed, causing moderate elevation of turbidimetric values.

individuals, made lipemic by a fat meal, cleared following the injection of heparin, and presented some evidence showing that in individuals suffering from arteriosclerosis, the sera were more resistant to clearing than that of normal persons.

Because of the current interest in heparin and co-factors, it was thought worthwhile to present evidence showing that the ability to develop "clearing factor" varies from person to person, and that this ability to manufacture a "potent clearing" factor is not necessarily related to the amount of heparin injected. In the course of a study of aging and arteriosclerosis, the fat tolerance test described by Moreton(5) has been used in many subjects.

The experience resulting from these studies facilitated the present one concerning the effect of heparin on lipemic sera.

Methods. Turbidity of sera was measured in a nephelometer constructed as nearly as possible like the one described by Moreton (5), using the standard of barium sulfate suspension in glycerin suggested by him. A *fat tolerance test* was carried out as follows: After an evening meal, low in fat content, the subject was asked not to smoke or eat prior to his test meal the following morning which consisted of a quantity of 20% cream calculated on a basis of surface area,[†] fruit juice, cereal, toast, jam and coffee. A fasting blood sample was obtained before the meal and at 3, 4, 5, 6 and sometimes seven hours afterward. Each blood sample was allowed to coagulate at room temperature, centrifuged, and the serum removed. The serum was recentrifuged, pipetted into cuvettes and the turbidity measured in the nephelometer. Peak turbidity in our patients, who were mostly over the age of 45 years, generally occurred between 3 and 4 hours after ingestion of the test meal. Total lipid content, phospholipids and total cholesterol were chemically determined on each serum sample. Since little change was noted in any of these values, except for neutral fat, the data were not included in this report.

Since it has been demonstrated that a large dose of heparin (50-100 mg) will indiscriminately clear the lipemic sera, of most individuals *in vivo,* it was decided to use a small quantity of heparin and, in an effort to achieve consistent results, relate the absolute amount given to the surface area of the subject.[‡] That the size of the dose selected was good for the purpose of demonstrating individual variability in the production of clearing factor becomes evident in the results (Fig. 1).

Results. As was to be expected from previous reports, the injected heparin caused a variable degree of clearing of the serum *in vivo* in each of the 5 subjects used (Fig. 1).

It also appeared that the serum of subjects L and CH developed more potent clearing factor *in vitro,* after incubation, than did the other subjects. While in our experiments maximal clearing of serum generally occurred within a period of 45 minutes or less after the injection of heparin, one subject (CH) exhibited maximal clearing only after a 75 minute interval.

TABLE I. Nephelometric Units of Turbidity. The sera mixtures of the 5 subjects, L, H, G, CH and CHG are indicated with the turbidimetric values before and after incubation.

Sera mixture[*]	Before incubation	After incubation	
6/11/52		30 min	90 min
L1 + L2	160	160	160
L2 + L3	158	96	78
6/25/52		135 min	15 hr
L2 + L3[†]	132	100	77
L2 + L4	159	136	100
H2 + L4	182	155	100
H2 + L3	141	91	91
G1 + G2	245	245	245
G2 + G3	232	195	195
G2 + L3	202	136	136
		150 min	15 hr
Ch2 + CH3	265	235	250[‡]
Ch2 + CHG3	280	255	240
Ch4 + CHG2	255	195	172
Chg2 + Chg3	285	255	240
Chg1 + Chg4	250	—	205

[*] The character of the sera used for these mixtures may be found in Fig. 1.

[†] This mixture contained 2 vol of serum L2 to one of L3 instead of the usual equal vol mixture. Note the maximal clearing despite the disproportionate mixture.

[‡] An increase in turbidity after prolonged incubation has been reported to occur occasionally.

Because subject L formed such potent clearing factor *in vitro,* his post-heparin sera was mixed with that of other subjects (Table I), and it was observed that in each instance potent "clearing factor" was formed after incubation. Similarly, the post-heparin sera of subject CH proved to be quite potent when mixed with that of subject CHg and incubated (Table I). It would seem therefore that those who cleared best *in vivo* developed more potent clearing factor *in vitro,* not only as concerned their own sera, but with regard to the sera of others as well (Table I).

Since subjects L and CH cleared their sera so well *in vivo* and *in vitro* while the others did poorly, an attempt was made to correlate the clinical histories with the results. Subject H was a 32-year-old physician, mildly obese, with a family history of vascular disease. Subject L was a paretic 50 years of age, while subjects CH and CHg were tabetics about 60 years of age. Subject G was a 57-year-old male with a history of cardiovascular disease and alcoholism. From this small group, however, conclusions cannot be drawn concerning the relationship between degree of formation of clearing factor and the disease process known to be present.

[†] 162 cc 20% cream/M² Surface Area.

[‡] Sodium Heparin U.S.P., 5 mg/M² Surface Area.

Discussion. These experiments have confirmed the work of Anderson and Fawcett(2) in which they demonstrated the presence *in vitro* of an antichylomicronemic substance in post-heparin plasma samples. However, in our experiments, incubation of the sera at 37°C appeared to be necessary to initiate clearing activity *in vitro.* It is to be noted that Anderson and Fawcett used much larger doses of heparin (50 mg) than those used in our experiments, or by Block *et al.*(4).

Several problems related to the size of the single injected dose of heparin need to be answered. For example, the larger dose of heparin used by Anderson and Fawcett(2) may make incubation of sera unnecessary to initiate "clearing activity" *in vitro.* Furthermore, large doses of heparin which not only cause greater clearing of sera *in vivo,* and perhaps for a longer period of time, may also initiate greater clearing *in vitro* after incubation with the formation of more potent "clearing factor." These problems are under investigation.

Since the work of Block and his associates (4) has indicated that the *in vivo* response to a single dose of heparin may be altered in vascular disease, further studies in patients with cardiovascular disease may also demonstrate differences in the degree of formation of "clearing factor" when compared with control subjects in the same age groups.

Summary. 1. By the use of a well-standardized fat tolerance test, it has been possible to demonstrate the clearing effect of small doses of intravenously administered heparin *in vivo.* Furthermore by use of these small doses of heparin, we have demonstrated that individuals vary in their ability to clear their own sera *in vitro* after incubation. It has also been shown that an individual who clears well *in vivo* will probably clear well *in vitro,* as in the experiments described. Apparently the potent "clearing factor" formed, as for example in subject L, was very potent for the sera of other subjects who formed less "potent clearing" factor under similar conditions.

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Premature Ovulation in the Domestic Fowl following Administration of Certain Barbiturates. (20056)

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Everett(1) and Markee(2) have recently reviewed evidence indicating that neurogenic activation of the hypophysis is essential for release of ovulation-inducing gonadotrophin not only in the rabbit, which normally ovulates following copulation, but also in the rat, a "spontaneously" ovulating species. Evidence of a 24-hour rhythm in the neural mechanism was adduced in the 5-day cyclic rat when ovulation was advanced 24 hours by progesterone(3), an action of the steroid which could be blocked by atropine or dibenamine (4). This 24-hour periodicity in the neural mechanism effecting release of hypophysial gonadotrophin was confirmed when Everett and Sawyer(5,6) showed that barbiturate sedation during critical hours of proestrus—a period of about 2 hours for the colony, even less for the individual rat(7)—delayed release of pituitary gonadotrophin by almost exactly 24 hours. If sedation were repeated over the same critical hours on the second day, release of the hypophysial ovulatory hormone was postponed by an additional 24 hours(6). Like atropine or dibenamine, Nembutal also blocked the release of ovulation-inducing hormones by progesterone in the rat(8).

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In hens under usual conditions of maintenance and lighting, the release of ovulation-inducing hormone (OIH) for the first or C_1 ovulation of a sequence occurs at a fairly definite time of day(9,10). A number of considerations, some of which have been noted previously(10), indicate the possible participation of factors outside the usually postulated ovarian-hypophysial relationships in timing of OIH release for C_1 ovulation. In the light of findings in the rat, it appeared not improbable that the regulatory factors in question might be of neural origin in the hen also. During the course of experiments on this problem an unexpected advancement in time of C_1 ovulation was found to occur following the administration of certain barbiturates. This effect in the fowl, which does not appear to have been described previously in any species, is reported on in the present paper. Some preliminary observations indicative of a complementary action between progesterone and those barbiturates favoring prematurity of ovulation are also noted briefly.

Materials and methods. The hens included White Leghorns, Rhode Island Reds and crosses between these breeds, all in first or second years of production. The birds were battery caged, with feed and water always available. Artificial lighting was maintained from 6:00 a.m. through 8:00 p.m. Time of lay of eggs was recorded on the hour from 8:00 a.m. through 4:00 p.m. for at least 2 weeks—usually much longer—before any hen was selected either for test or control purposes. *All tests* were carried out for effect on the C_1 follicle. The hens were laying sequences of 2 or more eggs, with but a single day lapse anticipated between sequences. Such hens, as a rule, lay the terminal egg of a sequence between 1:00 and 4:00 p.m. Release of OIH for C_1 ovulation of the next sequence takes place later than 10:00 p.m. of the same day (9), and C_1 ovulation occurs between 4:00 and 7:00 o'clock of the following morning (9,11), the mean time falling not far from 6:00 a.m.(11). Barbiturates were administered in single doses between 2:30 and 4:30 p.m., thus some 12 to 16 hours before the time of normal C_1 ovulation, or at least 6 hours before the normal release of OIH. *Prematurity of ovulation* was established, in most tests, by digital palpation *via* the cloaca at appropriate intervals until the egg consequent upon ovulation had attained either the fully plumped condition or the early stages of shell formation. A total of more than 150 uninjected and carrier injected control hens were subjected, from time to time, to exactly the routine practiced on test birds. The reliability of results based on palpation was further assured by autopsy of both control and barbiturate-treated hens. The *barbiturates* used and preparation for injection follow. Barbital sodium, 200 mg/ml distilled water. Phenobarbital sodium, 100 mg/ml distilled water. Diallylbarbituric acid (Dial),[†] 100 mg/ml propylene glycol; also "Dial with urethane," Dial 200 mg, urethane 800 mg and monoethylurea 800 mg/2 ml ampul solution. Calcium ethylisopropylbarbiturate (Ipral),[†] 20 mg/ml distilled water. Pentobarbital sodium (Nembutal), 60 mg/ml distilled water; also used as prepared solution, 60 mg/ml containing 20% propylene glycol and 10% alcohol. *Dial* was administered intramuscularly and subcutaneously, with little difference in result; the remaining barbiturates were injected subcutaneously. Where a range of dosage levels is given in Table I, the great majority of tests was run at the highest level noted. In terms of *duration of depression,* phenobarbital and barbital have been classified as long, Dial and Ipral as intermediate,

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[†] Dial and progesterone were supplied generously by the Research Department of Ciba Pharmaceutical Products, Summit, N. J., and Ipral by E. R. Squibb and Sons, New Brunswick, N. J.

TABLE I. Incidence of Premature C_1 Ovulations Following Administration of Barbiturates.

Barbiturate	Dosage, mg/kg	No. hens inj.	Premature ovulations No.	%
Barbital sodium	200	84	2	2
Phenobarbital sodium	50–150	38	0	—
Dial	80–100	57	16	28
Ipral	50–100	52	15	27
Nembutal	60	15	2	13

and Nembutal as short acting(12). Phenobarbital and barbital exhibit lengthy induction periods(12). Probably the same order of effects holds in the hen, but observations are limited. The action of barbital in fowl is to be noted, however. This drug was shown by Koppanyi, Murphy and Gray(13) to cause (at 225 mg/kg) a profound narcosis from which the birds do not recover, though they may survive for days under forced feeding. The drug is not metabolized and is very sparingly eliminated. The *induction of ovulation* with progesterone has been described elsewhere(14). In the tests described below, crystalline progesterone[†] was dissolved in corn oil (Mazola) and injected subcutaneously in breast regions, always distant from the site of barbiturate injection when used in conjunction with the drugs.

Results. The incidence of premature ovulations following the administration of barbiturates is recorded in Table I. There can be no question concerning the result with Dial and Ipral, since more than a fourth of the hens included in the totals were autopsied before ovulation could have occurred normally, as was confirmed by simultaneous autopsy of control birds. Nembutal probably belongs with Dial and Ipral as an effective agent in view of its action with progesterone (noted below). The exceptional number of hens treated with barbital to yield only 2 premature ovulations calls for a word of explanation. In preliminary tests to ascertain appropriate dosage levels, 1 of 4 hens receiving 200 mg/kg ovulated prematurely. The 16th hen injected thereafter did the same. In view of the lasting narcosis induced in fowl by barbital, tests were continued; none of the additional 64 hens ovulated prematurely. The results with phenobarbital were entirely negative.

Both autopsy and palpation records indicated that the interval from administration of barbiturates to premature ovulation was somewhat longer and more variable than is the same interval following the injection of luteinizing preparations at optimal dosages. If ovulation follows OIH release under barbiturate treatment by about the same interval that it follows the intravenous injection of luteinizing preparations, some 2 to 4 hours must elapse between injection of the drugs and release of OIH.

The failure to obtain a greater incidence of premature ovulations than is recorded in Table I cannot be attributed in most instances to disruption of the normal processes of OIH discharge or of follicular rupture. Of those hens which did not ovulate prematurely in response to treatment with barbital, phenobarbital, Ipral or Nembutal, 85 to 90% ovulated at the normally expected hour. Only a little better than 60% of the Dial-injected hens not ovulating prematurely did so at the normal hour. The early appearance of follicular regression in some Dial-treated hens subjected to autopsy after failing to ovulate either prematurely or at the normally expected time was suggestive of OIH discharge at subovulatory levels(15), but on this point the evidence is inconclusive. The results, taken altogether, confirm and extend the findings of Bastian and Zarrow(16) on the failure of Nembutal to block ovulation in the hen.

When it became clear that some barbiturates favored prematurity of ovulation, it seemed reasonable to expect a complementary or additive action between such barbiturates and progesterone. To test this supposition, progesterone was given at a nearly minimal ovulation-inducing level 30 to 40 minutes following the administration of Dial in one experiment, Nembutal in a second. The results, given in Table II, indicate a definite mutuality of action between each of the barbiturates and progesterone. Preliminary experiments have given some evidence, however, that the ovulation-inducing capacity of progesterone is at least partially reduced by phenobarbital; it will be recalled that no ovulations were recorded following the ad-

TABLE II. Enhancing Action of Progesterone with Dial and with Nembutal.

Treatment	No. hens inj.	Premature ovulations No.	%
Dial, 100 mg/kg	16	3	19
P,* .1 mg/hen	14	0	—
Combined	21	12	57
Nembutal, 60 mg/kg	15	2	13
P, .1 mg/hen	9	1	11
Combined	20	6	30

* P = Progesterone inj. 30-40 min. following administration of barbiturate.

ministration of this drug. The investigation of these varied relations is now in progress.

Discussion. The main objective of this report has been to establish the fact that C_1 ovulation may occur in a significant proportion of hens following the administration of certain barbiturates. The complementary action of such barbiturates (*i.e.*, those facilitating C_1 ovulation) and progesterone at near-minimal ovulatory levels strongly reinforces the direct evidence. In view of these findings, it is not surprising that the same barbiturates fail to block either normal or progesterone-induced ovulation, as Bastian and Zarrow(16) have previously reported of Nembutal.

There are no *a priori* grounds for supposing barbiturates or their metabolic derivatives can effect ovulation prematurely by direct action either on the ovarian follicle or on the anterior pituitary body. Without summarily dismissing these possibilities, the more probable supposition may be that the anterior pituitary releases OIH in response to neurogenic factors operating over a humoral pathway, such as Harris(17) has described in other connections. Drager(18) has described the required architecture, noting the absence of nerve fibers into the pars distalis. It is not likely that depression *per se* constitutes the immediate neurogenic condition for adenohypophysial activation, for the proportion of premature ovulations should then have been higher with at least some of the drugs employed. Neural excitation following a characteristic duration or intensity of depression, preceding depression (barbital?), or originating out of more complex conditions might of course be postulated. The variable interval from injection of the drugs to ovulation becomes understandable in these terms, as does also the failure to obtain premature ovulations with phenobarbital, in appreciable proportions with barbital, or in uniformly high proportions with even the more effective barbiturates. The mutual action expressed between progesterone and effective barbiturates and the apparent absence of this effect with phenobarbital are likewise consistent with the postulated secondary appearance of neural excitation.

Although the findings presented in this paper can thus be rationalized in the suggestive, if somewhat general, terms of neural excitation, proof of such an hypothesis obviously will require data of a different order than are now at hand.

The complementary action of ovulation-facilitating barbiturates and progesterone—even if only a summation effect—is of more than incidental significance in view of the reported occurrence of progesterone in the plasma of the hen(19). Variation in the blood level of endogenous progesterone might thus become an important factor in the variability of reaction, and possibly also in timing of response to a given barbiturate. The relative small quantity of progesterone employed emphasizes this point: if all the injected steroid were taken into the blood stream without loss the blood concentration of progesterone would be increased by no more than 0.75 μg/ml. Inasmuch as the steroid was given subcutaneously, in oil, and the rate of disappearance from the blood stream is known to be high(20), the actual change in blood level was probably considerably less than 0.75 μg/ml.

Selye(21) has shown that a number of steroid compounds, among them progesterone, desoxycorticosterone acetate and testosterone, are effective anesthetic agents in the rat; progesterone, at least, acts similarly in the immature fowl(11). Like progesterone, desoxycorticosterone acetate and testosterone induce ovulation prematurely in the hen(11). The results reported on here demonstrate that this rather remarkable association of anesthetic and ovulation-inducing effects in fowl is encountered also with certain barbiturates.

It may be noted in this connection that Hertz, Allen, Tullner and Westfall(22) have recently remarked upon the association of anesthetic and endocrine properties in the non-steroid compound Amphenone "B".

Summary. Ovulatory reactions of the first follicle of the hen's ovulation sequence to barbiturates, and to barbiturates with progesterone, are described. Ovulation was found to occur prematurely in a significant proportion of hens following the administration of some barbiturates (Dial, Ipral and Nembutal), rarely with barbital, and not at all with phenobarbital. Near subovulatory dosages of progesterone acted synergistically with two of the barbiturates (Dial and Nembutal) which themselves effect ovulation prematurely, an effect which was not found when progesterone was similarly administered with phenobarbital (preliminary observations). These several observations are consonant with the supposition that prematurity of ovulation following the administration of effective barbiturates results from neural excitation secondary to depression, excitation effecting over a humoral pathway the release of ovulation-inducing gonadotrophin from the anterior pituitary body.

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New Hemophilia-like Disease Caused by Deficiency of a Third Plasma Thromboplastin Factor.* (20057)

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(Introduced by W. Antopol.)

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Hemophilia has been generally recognized as a hereditary disease characterized by the deficiency of a plasma factor which reacts with platelets to form thromboplastin(1-3). This factor, designated as anti-hemophilic globulin (AHG), is essential for the normal conversion of prothrombin to thrombin(4,5). Recently, evidence for a second plasma thromboplastin factor has been presented on the basis of 2 reports of male patients with hemorrhagic disease and coagulation findings similar to hemophilia except that the blood of these

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TABLE I. Matching Experiments Performed on Mixtures of Whole Blood of Patients with Anti-Hemophilic Globulin (AHG), Plasma Thromboplastin Component (PTC) and Plasma Thromboplastin Antecedent (PTA) Deficiencies.

Exp. 1	B.Y. (Family A, PTA deficiency)	cc	2	1	0
	P.B. (AHG deficiency)	”	0	1	2
	Clotting time	min.*	24	5	62
	Serum prothrombin time	sec.†	15	24	12
Exp. 2	D.R. (PTC deficiency)	cc	2	1	0
	P.B. (AHG deficiency)	”	0	1	2
	Clotting time	min.	47	5	85
	Serum prothrombin time	sec.	8	35	10
Exp. 3	D.R. (PTC deficiency)	cc	2	1	0
	B.Y. (PTA deficiency)	”	0	1	2
	Clotting time	min.	35	9	25
	Serum prothrombin time	sec.	12	37	12

* Measured in glass tubes 13 × 100 mm at 37°C.

† Determined by the one-stage method of Dreskin(8).

patients mutually corrected the clotting defect in "true" hemophilia(6,7). For the past 3 years, we have been following 3 patients in one family, who have also had a hemophilia-like disease which was mutually corrected by hemophilic blood. Matching experiments were performed upon these patients (Family A), an unclassified hemophiliac (D.R.), true hemophiliacs and the patient reported by Aggeler, White *et al.*(6). The results provided conclusive evidence that there are 3 different plasma thromboplastin factors or precursors which can account for at least 3 types of hemorrhagic disease resembling hemophilia. The factors are AHG, plasma thromboplastin component (PTC) described by Aggeler(6) and the factor described in this paper and tentatively designated as *plasma thromboplastin antecedent* (PTA).

The patients in Family A, a 50-year-old maternal uncle (A.A.) and 2 nieces (B.Y. and M.W.), aged 29 and 25 years, have shown since childhood only slight to moderate evidences of hemorrhage, marked chiefly by bleeding after tooth extractions. Their clotting times have ranged from 15 to 29 minutes (normally 5 to 11 min.) and the serum prothrombin times have been between 12 to 17 sec.(8). Clotting results were the same for the uncle and both female members of the family. Other studies including platelet count, clot retraction, prothrombin time, tourniquet test and bleeding time have been normal. No anticoagulant was present. Matching experiments revealed that the 3 members of the family did not correct each other and, hence, had a similar clotting defect. Further experiments revealed that these patients' bloods mutually corrected bloods from patients presumed to be true hemophiliacs as evidenced both by the clotting time and serum prothrombin time (Table I, mixture of bloods from B.Y. and P.B., a "true" hemophiliac).

Patient D.R., a 4-year-old male, has had intermittent episodes of severe bleeding and subcutaneous hematomas dating from circumcision in the first week of life. There is no family history of bleeding. His clotting time has ranged from 35 to 47 minutes and serum prothrombin time from 9 to 13 sec. Other routine clotting tests have been normal. An anticoagulant was not present, and the patient's blood mutually corrected blood obtained from hemophilic patient P.B. (Table I). Additional experiments then revealed that patients D.R. and B.Y. (Family A) corrected each other (Table I). A number of experiments using other hemophiliacs and employing both blood and plasma mixtures have consistently yielded the same results. Experiments performed with plasma[‡] of the patient reported by Aggeler, White *et al.*(6) revealed that Aggeler's case was identical with our patient D.R. (Table II). Thus, Family A represents the third type of deficiency which has been designated as plasma thromboplastin antecedent (PTA) deficiency.

In addition to matching experiments, it appears that the 3 types of hemophilia-like disease or plasma thromboplastin factor de-

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[‡] We wish to thank Drs. Sidney White and Paul Aggeler for providing the plasma from their patient.

TABLE II. Mixture Experiments Using the Recalcified Plasma Clotting Time Performed on Aggeler's Case of PTC Deficiency, Patient D.R. and Patient P.B., a True Hemophiliac.

P.B.	cc	.2	.15	.1	.05	0
Aggeler's case	"	0	.05	.1	.15	.2
$CaCl_2$ .025 M	"	.2	.2	.2	.2	.2
Plasma clotting time	min.	18	5	6	5	22
Serum prothrombin time	sec.	8	35	25	20	11
D.R.	cc	.2	.15	.1	.05	0
Aggeler's case	"	0	.05	.1	.15	.2
$CaCl_2$ .025 M	"	.2	.2	.2	.2	.2
Plasma clotting time	min.	23	20	17	15	17
Serum prothrombin time	sec.	7	13	15	15	11

TABLE III. Effect of Normal Serum, $BaSO_4$-Treated Normal Plasma and PTC Fraction on Plasma from Patients with AHG, PTC and PTA Deficiencies. Results represent recalcified plasma clotting times in min.

	cc	AHG def., .1 cc plasma	PTC def., .1 cc plasma	PTA def., .1 cc plasma
Normal serum	.1	10.5	3	4.5
$BaSO_4$-treated plasma	.1	4	15	5.5
PTC fraction	.05	10	3	3.5

ficiencies can be readily distinguished as follows: AHG deficiency (true hemophilia) is corrected by $BaSO_4$-treated normal plasma but is not corrected by normal serum(9); PTC deficiency is not corrected by $BaSO_4$-treated plasma but is corrected by normal serum(6); PTA deficiency is corrected by both $BaSO_4$-treated plasma and by normal serum. PTA deficiency was also corrected by the lyophilized PTC fraction obtained from Drs. Aggeler and White(6). (Table III).

Clinically, certain distinctions can tentatively be made among the 3 types of deficiencies. PTA deficiency can occur in both sexes and is inherited. The hemorrhagic manifestations appear to be relatively mild and the clotting times are not very prolonged, ranging from 15 to 29 min. PTC deficiency, as exemplified by 2 proven cases, occurs in males without a family history of bleeding. The hemorrhagic manifestations are more severe and the clotting times longer than in PTA deficiency. Cases previously designated as hemophilia have shown a wide range in degree of hemorrhage and prolongation of clotting time(10,11). It is possible that some of these differences can be attributed to the type of clotting defect as contrasted with explanations based upon differences in degree of a single clotting defect. Various combinations of AHG, PTC or PTA deficiencies in a single patient or the occurrence of specific anticoagulants could account for different degrees of the hemorrhagic syndromes now classified under hemophilia.

Summary. 1. A third type of hemophilia characterized by a deficiency in a plasma thromboplastin factor other than anti-hemophilic globulin and plasma thromboplastin component (PTC) has been identified in 3 patients in one family, a maternal uncle and 2 nieces. 2. These patients reveal a mild hemorrhagic disease, and their clotting times are only moderately prolonged. 3. This newly described clotting factor, designated as *plasma thromboplastin antecedent* (PTA) is present in $BaSO_4$-treated normal plasma and normal serum.

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